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PREFACE

Effective communication has a beginning, a middle, and an end. These three stages are represented in the three volumes of *Annual Review of Microbiology* that are in some stage of preparation at any one time. As this is being written, contributors to Volume 40 are being selected, authors are writing reviews for Volume 39, and edited manuscripts for Volume 38 are being typeset. Each volume starts its second life when you, the reader, open its covers.

Preparation at all stages is kept in balanced motion by our talented production editor, Dave DeRoche, whose eye for troubled syntax has made good writing even better. Editorial responsibilities have been organized by a superb administrative specialist, Anne Harrison. The difficult charge of presenting concisely a unique perspective on a single area of research has been met well by all the authors of Volume 38, and we thank them for their efforts. Selection of topics for this volume was guided by the collective wisdom of our continuing editorial board and by three helpful guest members: George Chang, E. Peter Greenberg, and Abdul Matin. We are fortunate that Paul Baumann recently has become an associate editor and that Albert Balows has graciously agreed to serve another term in the same position.

The production schedule allows us to see two years into the future, and I would like to herald two changes that will appear in Volume 40. The first is increased coverage of parasitology. The second is the inclusion of article titles in the references that follow each review. As always, readers' comments on any topic are most welcome.

L. NICHOLAS ORNSTON
EDITOR

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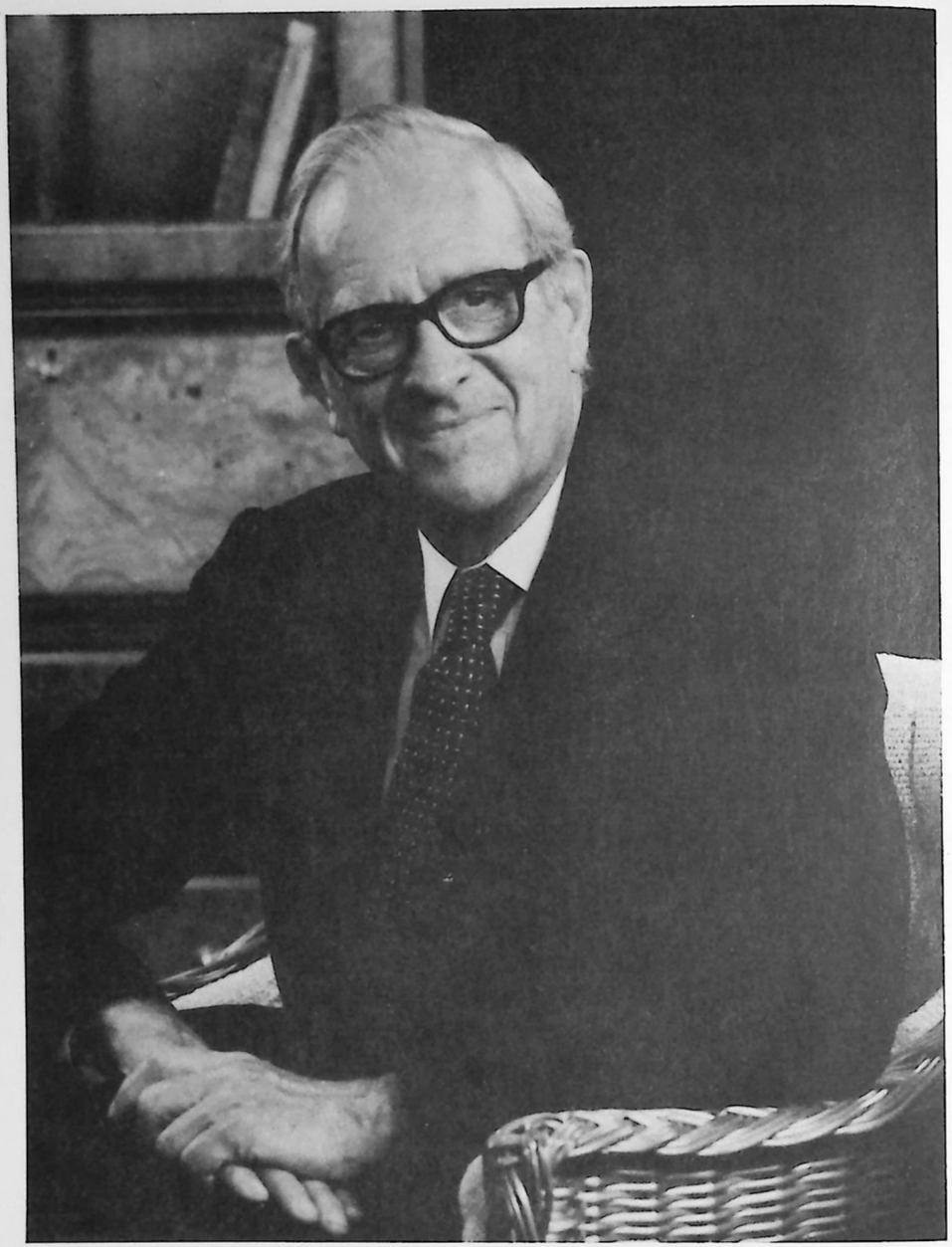
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LEARNING

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To My Children and Other Teachers

1 WHENCE AND WHITHER

E. B. White characterized writing as "an act of faith, nothing else. And," he added, "it must be the writer, above all others, who keeps it alive—choked with laughter or with pain." The laughter is here, and the pain; may both show. Much laughter emerges from the pool of memories reflected by perusing the numbered publication list: titles, coauthors, dates, theses, and the many long close associations. The pain derives from omissions, inaccuracies, and the slow anastomosis of thought. May these form true mosiacs, to be redressed another day from an alternate streamhead. Each painting, perfect in the mind's eye, may deflect en route to appear less so on canvas, according to the great French painter A. M. Guérin V. So it is with writing. From Carl Becker, a Cornell professor of modern European history, I learned: "A thought expressed to the extent of one's talent merits repetition." Thus, later in this chapter reside some words of personal and professional events taken from "Lessons in Learning"

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(45) written in tribute to Severo Ochoa on the occasion of his seventieth birthday. From an alternate vantage, *Experiences in Biochemical Perception* (88a) may expose a more recent true image.

The Dakota Territory, settled after the Civil War by immigrant pioneers, attracted my maternal grandparents, Morris Shea and Rebecca Copley Shea. Together they filed a Civil War veteran's preference homestead claim near Miller, South Dakota. There my mother Anna, the eldest of three children, was born on February 16, 1883.

My paternal ancestors had arrived in Philadelphia in the 1700s, originally from the Atlantic coast of Europe. Franklin Gunsa*u*lus, my grandfather, and my grandmother, née Rose Bates, of English descent, migrated westward to the Chicago environs and later to Clinton, Iowa, where my father, Clyde, the second of four sons, was born on the Fourth of July, 1878. With the opening of the Chicago & Northwestern Railway, grandfather moved the family westward to the terminal station: Blunt, South Dakota. Settler claims were filed for grandfather and the four sons in South Buffalo Township, Sully County—on the open prairie, a few miles from the Missouri River and 15 miles from the nearest town. According to mother, I was born there "in a farm cottage, with the help of a midwife, on June 29, 1912." My birth was not recorded with the state until my enrollment at Cornell required a certificate.

My earliest memories, no doubt reinforced by parental tales, are of rolling grassland reaching to the horizon, barns, livestock, a windmill, and a duckpond supplied by an artesian well. Beyond the farmyard fence were coyotes, and within were cottontails and numerous turkeys and chickens. Father's aged riding horse, persuaded with sugar, took me and my older sister to a one-room school house, two and a half miles across the pastures. Evangeline McArthur, a sweet young teacher, also arrived on horseback to instruct 17 of us in various grades—until the "flu epidemic" canceled school after Christmas.

That was my first-grade education. My parents always expressed an interest in education; many times my father had told me that he had finished the fifth grade and my mother the tenth. Largely self-educated, he learned readily. He was also mechanically gifted, self-reliant, imaginative, and at home in the outdoors. He farmed the homestead section; later, as a "sidewalk farmer" (town resident), he earned a comfortable income from flax grown on 10–15 quarters (1500–2500 acres) of rented native prairie. He was lost in a threshing accident while harvesting alfalfa for seed during my senior year in high school. From him I learned to enjoy the outdoors and to appreciate whatever I had. I also learned that money has value only when used for the benefit of family, friends, or self.

My mother earned a teacher's certificate and taught rural school in South Dakota and later in Hood River, Oregon, before returning to South Dakota. After a long life, "enjoyed to the full" in a world of change, she passed away

gently in sleep a few days short of her eighty-seventh birthday. In Brookings she worked as a bookkeeper in a bank; after the bank closures of the 1930s, she worked in the County Clerk's office. For many years she also prepared the annual books of the city assessor. On the farm she raised chickens, capons, and turkeys that were frozen in barrels and shipped for sale to Chicago. Starting from egg, chicken, and turkey savings she accumulated and managed houses and enjoyed a wide circle of friends and local business associates to whom she served as counsel. When I enrolled in college I appreciated her response to my progress, and when I wanted to go to Cornell, she assisted without regard to sacrifice.

My days in Sully County terminated in the fall of 1919 with the sale of the family farm, with all machinery and stock, to a fine group of Mennonite migrants. Our family moved briefly to Blunt, but soon I was sent by train to my grandmother in Brookings, also in South Dakota. After World War I, Grandmother Shea, widowed, moved to Long Beach, California; her vacated home in Brookings was purchased by our family. Here I lived with my mother and two sisters from the second grade through high school and two years of college.

The Brookings grade school system reflected the community, with many teachers of first-generation Nordic descent. The English teacher often was assigned to teach mathematics as well. The books were clear; we could stay well ahead of class requirements and bedevil the fearful teacher. I learned grammar mostly in the seventh and eighth grades from Miss Enstiness, a full-200-pound disciplinarian with a ruler: "Everyone up to potential or else." My mother bought a trumpet for me and I marched in the band. I tried various athletics, in a desultory way, without marked success. I opted rather for dramatics, music, and participation in a Boy Scout troop that survived often without senior leadership. Through junior and senior high school I spent much time independent of supervision. Soon I was permitted my own key to the wood-working shops. Scheduling conflicts arranged between Latin and science classes encouraged most boys to neglect languages, including English.

Memories of several challenging teachers remain from this period. Mathematics was traditionally taught by the football coach; fortunately ours, Bob Cofey, was an electrical engineering graduate and a first-rate teacher and person. Chemistry then physics were offered by Marcus Aurelius Chase, a scholar of curiosity and kindness, with friendly piercing eyes, who questioned and listened, especially in laboratories. I retain an especial gratitude to Esther Test, the senior English teacher who served also as dramatics coach; her demanding methods brought quality, success, and reflection. She would say, "In a part on the stage, be always in character of name, lines, situation, and context." Due to her drama teaching and the resulting support of Esther Taskrude and Georgia Tate, there and later in college I was assigned more leading roles than others. I also owe much—for stimulation in curiosity and in

learning, from comments and example, to enter into the wider world—to: Marcus Chase and later Laurence Carruth, who served as scoutmasters, and among the troop committeemen, William Powers, the college librarian, and Ward Miller, the college botany professor and a Chicago Ph.D. of the Coulter, Barnes & Cowell era.

These experiences, coupled with the paucity of jobs facing my buddies graduating in engineering during the Great Depression, stimulated me to enroll as a chemistry major when I entered the South Dakota State College in the fall of 1931. Additional contact developed with Ward Miller; his lectures were stimulating, direct, and perceptive. His remarks and comments as he frequented the laboratory refined my methods. During the summer after my first year of college on many an afternoon along a local creek in the shade of the willows, his witty questions, accompanied by a flick of an eyebrow and punctuated by his large cigar, brought detail to my seeing nature as I sorted through a collection of local flora with the aid of Grey's Manual. In two years of chemistry, Ed Bennowies, a student of Julius Stieglitz at Chicago, taught from inorganic examples the basic principles of molecules and the separation and properties of the elements. He also introduced some organic chemistry. Lester Guss, a Minnesota Ph.D., taught rudiments of quantitative and chemical theory. In physics, Professor Mathews, the department head, taught with a kindly penetration. In mathematics, Dean Brown, the senior dean and recurrent interim president, offered sections of advanced calculus. With droll humor, he would graciously surrender a piece of chalk to an inattentive student, including myself, to work out that day's lesson at the board. At his examinations, hourly or final, one turned in a paper early but once—the remaining time would be spent at the blackboard trying to solve less easy examples.

Thus during the Depression two pleasant years were spent at this small rural college staffed by returned natives. They provided an enlightening exploration of fundamentals, with time for a varied and free existence. My intent to enroll in biological chemistry at Minnesota, with Ross Gortner, was diverted by Laurence Carruth with the remark, "Why go from a small Norwegian school to a larger one? Why not go away? There are places really different where one can learn new ways."

I enrolled in Cornell as a college junior. Larry Carruth became my roommate as he began his Ph.D. studies and we shared an apartment with four biology graduate students. At this turning point, I soon became aware, as Carl Becker put it, that "In the tradition of freedom and responsibility, Cornell has acquired a reputation and something more: a character." The freedom fit well on an individual who had been turned loose through his early years in a broad country under minimum supervision, and who had acquired a well-developed habit of shifting for himself and viewing occurrences as natural phenomena. I soon found that my background of two college years of fundamental quantitative

science, mathematics, chemistry, physics, botany, and English, drawing, dramatics, and summer school psychology, plus my growing tendency to give my best, within reason, to whatever passed before me, had provided an education superior to that of many of my new colleagues.

For a master's degree, two years were spent on a series of observations that did not develop into a defined problem. However, I developed manipulative skills and received financial support as a teaching assistant in general microbiology laboratories. Room and food were provided by keeping a university building locked and the windows closed against the quick and frequent thunderstorms.

For a Ph.D. I moved to Dr. J. M. Sherman—Uncle Jimmy in graduate student vernacular—and his beloved streptococci for three years of unsupervised learning from the lactic acid bacteria and from Uncle Jimmy, just as he had learned from Lore Rogers in an earlier day. In parallel, I became undergraduate advisor to the 150 students per year majoring in bacteriology. My graduate days were completed in February of 1940; I accepted an instructorship at Cornell.

A window of major impact had opened the previous summer when I attended an international congress of microbiology in New York. Among the giants of modern biology present were the legendary Marjorie Stephenson, C. B. (Kees) van Niel, and A. J. Kluyver. Van Niel came back to Ithaca with our professor, Otto Rahn, a converted physicist from Kiel who insisted on precision, graphic method, and mathematical analysis. Dr. Rahn invited me to spend a day in the gorges of Enfield glen with him and Kees van Niel; Kees was everywhere in excitement over our numerous ecological nitches in nature.

At Cornell, a place to apply chemistry, to pursue biology, to search for causes among reactions and molecules was taking shape. The work, the laughter, the newness—recognition of alternatives was opening; my learning was not lost. The following summer, spent at the University of Wisconsin, brought access by early fall to the band of European biochemists who carried to our shores the sophistication to develop the next decades.

The years of learning and of science have been interlaced and enhanced with the growth of seven children, who arrived at various stages. Gene, Glen, Ann, and the twins Rich and Rob were born when we were at Cornell. Over the years their mother, Merle, and I grew apart; in 1950, as the Indiana years drew to a close, we were divorced. I set off alone for an interim learning period with Severo Ochoa in New York. In 1951 Carol Foust, then an associate professor at Cornell, and I were married. We spent many years of productive science together at Illinois, rearing my and our children (Tina and Kris), until Carol's unexpected death early in 1970. Dorothy Clark, a professor of music (voice) and I were married in the fall of 1970 and spent 12 pleasant years together, raising Tina & Kris. In the older children she found five new friends and in their spouses additional joy; in her calm warmth each of us gained much valuable

love and learning. We lost her to heart problems in the fall of 1982. To Kris, now graduating from Cornell, to the other children, now professionally established, and to the ladies who helped to raise them, I owe a large portion of my education.

2 LESSONS FROM THE LACTIC ACID BACTERIA

J. M. Sherman's streptococci, with nutritive requirements akin to mammals, were an enriching encounter in the early 1930s. An abundant, classically ordered collection of cultures provided a wealth of biological exploration for entry into the chemistry of microbic processes. This kindly mentor-counselor arrived at our laboratories and offices on a frequent and predictable schedule, bringing professional science, lore, gossip, and personal vignettes, along with recurrent nudges for growth of the "young giants," as he generously, if erroneously, cast us. Through teaching in general laboratories and lecturing with Uncle Jimmy in the applied counterpart, with emphasis on the properties of organisms, we learned the rudiments from test tubes, litmus milk, enriched broth, and agar. Coping with an inevitable array of "fastidious" organisms taught us strain manipulation and maneuver for survival, readily transferrable to other systems. From test tubes and empiricism to dynamics, equilibria, and chemical regulation, the trail proceeded well. Otto Rahn's bacterial physiology plus his lovely and stimulating book (93a) furnished the organizational base of cells, growth, and death upon which we were to later spin our webs. Soon I was permitted to introduce, for senior and graduate students, a course on "the chemistry of microbic processes." With 18–20 senior majors and a smaller number of graduate students, I shared the challenges, excitement, and mysteries. Slowly we learned to dispell and recast the folklore in hypotheses, quantitation, chemical reactions, and energy terms; models were formed and tested. Genetic organization, control, and exchange gathered more slowly, and formed first on a fermentation-reaction base.

My working experience centered on the enterococci, Rebecca Lancefield's serological group D, a few orange-pigmented group B strains, and *S. lactis,* the primary organism of milk habitat. Only later the heterofermentative cocci of the leuconostoc group gave us a new fermentation pathway, in the formation from hexose of equamolar amounts of lactate, ethanol, and CO_2. We learned to feed bacteria and, by nutrient restriction, to handicap differentially their growth and metabolism. After Karstrom we learned the rudiments of how to vary metabolic patterns and their abundance; by experience, we also learned to optimize cell growth, to measure metabolism by cell suspensions, dried preparations, and extracts, and to simplify the extracts by separations and deduce single catalyst parameters.

We entered chemical microbiology in the 1940s as we at Cornell and others

evolved the methods that liberated many from the taboos of permeability and brought the microbes to biochemistry as tools with the power of enzyme abundance and catalytic selectivity. Energy yield a la Elsden illuminated routes of substrate turnover and, later, cofactor-reaction cycles during catalysis. Single enzyme and multienzyme assemblies soon appeared in our publications.

The chemistry of lactic fermentations was enriched by our access to the treatise of the Danish microbiologist, Orla-Jensen (87), visualizing substrate structures, product yields, and mechanisms; much did not rhyme with the Sherman lore. The phenotypes could not withstand redox-balance analysis, could not yield exclusively lactate as from hexose. For example, other products must appear from glycerol with two excess hydrogens per triose (39) and from the less reduced malate of a redox equivalent to lactate plus CO_2. The calculations led to prediction, then detection of modified product yields, followed by identification of pathways, intermediates, individual reactions, the organization of enzyme patterns, the alternatives, and optimal reaction conditions for each.

We departed from the era of streptococci in balanced anaerobic growth reactions in an approach to external electron acceptors. Enterococci grew on glycerol in the presence of yeast extract, fumarate, or simply O_2 (e.g. glycerol + fumarate $\rightarrow$ lactate + succinate + cells). The fumarate-succinate redox couple could be replaced by aerobic growth. Increased growth, signifying energy release via a flavoprotein-O_2 couple, was soon recognized. Uncle Jimmy was first perplexed by the permissiveness of streptococcal cells to external O_2, then fascinated, and soon educated. His scoffing toward Orla-Jensen as "a strutty little peacock" receded as the underlying biology of metabolic studies recognized the correlation of "physiological phenotypes" with the serological ones of Rebecca Lancefield. The cellular versatility of lactic acid bacteria was extended in a variety of papers with students or by myself, and a smaller number with Dr. Sherman. The latter carried, true to his philosophy, careful editorial refinements as part of the learning process for "possibly bright" students.

Much insight derived from the Wisconsin biochemistry lecture outlines of Peterson & Johnson and from the Iowa State equivalent. The Wisconsin data reflected an early interest in the clostridial butanol-acetone solvent fermentations and the coliform data of William Mansfield Clark; the Iowa State papers stressed the Harland Wood discovery via the propionic bacteria that CO_2 fixation by heterotropic organisms is a vital part of nature. These were strong sources of excitement, enlightenment, and encouragement.

A high point for us developed from the innovation of a $400 fellowship for Alex Wood, a student of Professor Blythe Eagles of the University of British Columbia. A trail of students was also initiated, including Jack J. R. Campbell, Roberts Smith, Bill Razzell, and Doug Gillespie. Alex promptly established

himself as a maverick interested in Lash Miller's growth factors; he began, but soon discarded redox measurements with methylene blue. Our initial detection of redox processes with the very simple Thunberg tube and redox-dye methods was soon replaced by the more quantitative manometric method of Warburg. David Hand joined the department as resident "biochemist" and acquired the first seven-manometer Amico "Warburg" because Dr. Sherman preferred to have him associated with the metabolic effort. Betty Chase, a bright, well-educated New Englander, had entered as a graduate student; for reasons now obscure, the group B streptococci were targeted for investigation. Their orange-red pigment may have seemed a likely candidate to impart oxygen tolerance, possibly metal-mediated respiration. A strain was selected that oxidized ethanol actively, with a stoichiometry indicating a four-electron reduction of O_2 to water, although H_2O_2 served also as a two-electron oxidant. The mechanisms were elucidated only ten years later as Mort Dolin isolated the array of DPNH oxidative enzymes from group D streptococci—a four-electron two-DPNH redox center for reduction of O_2 to water, plus a single DPNH nucleotide system for H_2O_2 formation and another for its reduction.

The O_2-mediated pyruvate oxidation aspect of the Fritz Lipmann 1939 Cold Spring Harbor paper with acetylphosphate generation by a lactobacillus caught our eyes and imagination. Was this a general system, even among the lactics, for an energy couple supporting added cell yields? Katherine Miller arrived, quickly found conditions for formation and expression of active pyruvate oxidation for the enterococcus *S. faecalis* (strain 10C1): a rich medium, active cell (AC), 1% each of tryptone and yeast extract, enough glucose to bring the pH below 5, and often, as buffer to enhance cell yield, 0.5% potassium phosphate. The high specific catalytic activity in these prokaryote cells soon gave an open sesame to enzyme studies, and later to the O'Kane explorations that revealed the pyruvate oxidation factor (POF) role as lipoic acid, the more general energy-coupled ketoacid-oxidative pathway and enzyme complexes in both prokaryotes and eukaryotes.

Alternate reactions of pyruvate catalyzed by the enterococci were revealed in fermentation balances on hexose in alkaline media and on organic acids, using varied nutritive restrictions. The partition function versatility included a shift from pyruvate reduction to oxidation and to the formation of formate plus acetate and ethanol. Charlie Niven noted a reference to formate production from glucose in alkaline medium. With Charlie, an apt and handy laboratory colleague, we promptly confirmed formate as a product of cells growing on glucose at fixed pH, with yield graded downward with pH; cell suspensions gave similar, although less marked, trends. The redox balances were preserved by equivalence of formate to the sum acetate plus ethanol (i.e. a coliform type fermentation in the lactics without formate lyase).

The organic-acid metabolism explored in lactic bacteria by Jack Campbell & Dex Bellamy (15) showed that both malate and citrate supported growth, with product yields suggestive of additional induced-enzyme pathways. The work was interrupted by the event at Pearl Harbor and Jack's urgent recall to his native Canada. After 1950, the studies of Bob Smith with isocitratase and Doug Gillespie with citrate metabolism reached the enzyme mechanism stage. Jack Campbell during a summer at Illinois documented an iron requirement for fungal aconitase.

A resolution to the heterolactic fermentation with "obligatory" equimolar yields of lactate, ethanol, and CO_2 occurred to me during an afternoon visit, with Seymour Korkes, to Martin Gibbs at Brookhaven. Martin had prepared ^{14}C-glucose molecules labeled only at carbon 1 or equally at carbons 3 and 4. After leaving Severo's laboratory in August of 1950 I ran the fermentations at Brookhaven with *Leuconocstoc dextranicum* (strain 39). C_1 appeared entirely as CO_2, C_3 as the ethanol methyl, and C_4 carboxyl to C_6 methyl in the lactate. At Indiana I mentioned to Ralph DeMoss as a possible thesis problem the elucidation of the heterolactate pathway, enzymes, and mechanism. Ralph found a fermentative Entner-Doudoroff fission differing only in the nature of the redox acceptors from the aerobic *Pseudomonas* reaction. The cells lack fructose bis-phosphate aldolase and derive CO_2 from pyruvate via a yeast-type carboxylase. As a sidelight, the glucose-6-phosphate dehydrogenase is NAD- rather than NADP-linked and still serves in glucose estimates by clinical analyzer.

Sherman's streptococci taught us much. As an add-on, which we also exploited, Snell & Strong in 1939 had equated bacterial growth factors with animal vitamins. Soon quantitative growth assays followed first with flavine, then with most B vitamins. Not sensing the imminence of Pearl Harbor, we were a happy band, quickly at work and deeply committed. Dr. Sherman, as a chance vehicle, accepted an assignment to seek a remedy for the spoilage of unripened cheese shipped from the US to the U. K. as concentrated protein source. The dietary results were attributed to amines, either simple or in peptides. Tyrosine crystals accumulated in cheddar cheese, implicating peptidases, from the cheese-ripening or "containment" bacteria. The questions were delegated to Bellamy and me; the manpower freeze mandated my retention at Cornell for the duration. This time, without travail, we were welcome to undertake the "active cell studies." Soon Bellamy found by the deficient-nutrition technique that optimum formation of tyramine from tyrosine required 20-fold the B_6 level required for mere cell growth. That we learned well from the lactic acid bacteria is documented by the Bellamy (11, 12) experiments and the subsequent ones of O'Kane (85, 86). These lessons are only now being relearned by the medical industry.

3 PYRIDOXAL PHOSPHATE, THE AMINO ACID COENZYME

An invitation to present a Harvey Lecture in the fall of 1949 provided an opportunity to summarize our discoveries of the cofactors of the amino and the ketoacid enzymes (40). One observation was alpha amino group activation by the 4-aldehyde form of vitamin B_6 after its conversion to the coenzyme, pyridoxal-5-phosphate. The other was identification of a new cofactor, the pyruvate oxidation factor, now known as lipoic acid, and its role in a unique energy couple disulfide redox and transfer reaction sequence.

Briefly, the lecture identified the working tenets in the use of microbial cells for the solution of general biological and biochemical problems. I also summarized our use of restricted nutritive levels to identify the metabolic function and coenzyme forms of the vitamins. In 1940 we were aware of the Lwoffs's association of the *Haemophilus* growth factor V with coenzymes I and II and the immediate restoration of oxidative capacity to cell suspensions, suggestive of the presence of one or more apoenzymes (82). We knew also of the determination by William C. Rose of the amino acid levels essential to man. To these we added the manipulation of growth physiology to maximize enzyme production, then apoenzyme formation in cells supplied suboptimal levels of nutritive essentials. The coenzyme forms were revealed in the activation of freeze-dried cell preparations, which also provided multiple short-term determination of quantity.

The amino acid functions of vitamin B_6 were clearly demonstrated in two papers with Dexter Bellamy. The first established the conditions of growth required to maximize the level of the tyrosine decarboxylase that we used as an indicator enzyme, the second produced the apoenzyme by limiting the level of B_6 during cell growth. A more active form of the vitamin B_6, termed "pseudopyridoxine," had been discovered by Esmond Snell and traced to the 4-aldehyde, pyridoxal, and the 4-amine, pyridoxamine. In response to our informing Snell of our lead, he provided two vials and reference to a note in press in the *Journal of Biological Chemistry*. We found that the aldehyde, pyridoxal, activated the apotyrosine decarboxylase in cell suspensions and with the lyophilized cells, ATP was required as well. The ATP requirement suggested to us a phosphate or nucleotide ester coenzyme. Synthetic efforts were begun. Notes to the *Journal of Biological Chemistry* in the two months following the Snell publication (100) carried our report of the role of pyridoxal (48) and the coenzyme as 5-phosphate ester (49).

The optimum pH for tyrosine decarboxylase, around 5, afforded direct measurement of CO_2 release. With access to synthetic pyridoxal and analytical methods of the Merck Research Laboratories supplied by Karl Folkers, I proceeded to phosphorylate pyridoxal under various conditions while Wayne

Umbreit followed the coenzyme yields with the dried-cell assay. Soon we identified pyridoxal-5-monophosphate as the coenzyme active in the amino acid reactions (64).

We quickly broadened the base of pyridoxal-phosphate functions to other amino acid decarboxylases and, with Avery Wood, to a series of alpha and beta elimination reactions, including serine and threonine deamination, the dehydratases, the similar desulfhydrase of the cysteine, and the liberation of indole and pyruvate from tryptophan by tryptophanase (113, 114). Shortly the pyridoxal–pyridoxamine phosphate interconversion was demonstrated by Doreen Jeffs (O'Kane) in the glutamic-oxalacetate transaminase (GOT). The pig-heart preparation of Green & Leloir was found to lose more than 90% of the activity during the first ammonium-sulfate precipitation, a result we traced to loss of the coenzyme (84a).

After our move to Illinois, Dick Sagers came from Brigham Young University with a problem he had developed with Jay Beck, concerning serine and glycine interconversions by *Diplococcus glycinophilus*. This proved to be a multicofactor, multienzyme complex initiated by a pyridoxal-phosphate reaction with the alpha amino group of either amino acid. Subsequent steps include tetrahydrofolate as a methylene group acceptor and transfer cofactor, and cocarboxylase and pyridine nucleotide (NAD) as cofactors to mediate the decarboxylation and redox processes. Later a heat-stable peptide requirement was traced to the presence of a lipoyl group (60, 76).

In summary, the deficient-nutrition technique, which we called music minus one, served to demonstrate the biological role of vitamin B_6 with the tyrosine decarboxylase apoenzyme. This provided an entree into a broad array of alpha amino acid formation and catabolic processes; thus the term, amino acid coenzyme.

4 POF, NOW KNOWN AS LIPOATE: A UNIQUE OXIDATIVE REACTION OF KETOACIDS

"Too many cofactors" greeted our announcement of a new catalytic essential of broad distribution for energy-coupled pyruvate oxidation. Subsequent experiments validated the occurrence of a generalized array of enzyme complexes, functional in ketoacid conversion throughout the animal and plant kingdoms. Between the initial discovery of the activity termed POF and the structure determination, about 2.5×10^5–fold purification from yeast (62) or liver residue afforded the recovery of about 10 mg of crystalline α-lipoic acid (95). The synthesis followed closely the cofactor isolation to provide final proof of structure and free the enzyme research of material restriction (72).

The growth conditions found by K. Miller (83) to produce maximum levels

of pyruvate oxidation in *Streptococcus faecalis* were included in explorations by Dan O'Kane for the cofactor requirements of several metabolic reactions. With superb biological insight he observed that abundant cell crops from a defined medium lacked pyruvate oxidative capacity found in cells harvested from the yeast tryptone medium (85, 86). Known vitamins and cofactors failed to restore activity, whereas tissue extractives from liver, yeast, and bacterial cells were rich sources in restoring promptly the pyruvate-oxidase activity. Commercial yeast extracts, and later aqueous liver residues from antipernicious anemia–factor preparation were found to be potent and inexpensive sources, available in quantity. The prompt response of cell suspensions was attributed to the presence of one or more apoenzymes. One to two mg of the lyophilized cells provided a 20-minute Warburg respirometer assay by oxygen uptake rate (55).

Fractionation of yeast extract on activated charcoal, with alcoholic ammonium elution and solvent extraction, provided but traces of the active material about 3000-fold concentrated. Thus we suspected a minute requirement indicative of a cofactor tightly bound to the enzyme(s). Later, Lester Reed of the University of Texas found that a covalent enzyme lysine-ϵ lipoamide was the active form. Recognizing the potency of the cofactor, we had enlisted the assistance of the Eli Lilly research laboratories to prepare the larger quantities of the active principal required to reveal the chemical identity. Other unidentified substances were soon found to share POF properties. One, the acetate-replacing factor for *Lactobacilli* reported by Snell & Guirard, was also under fractionation at Eli Lilly for Lester Reed. Their pointed recommendation for us to join forces was followed, and the fractionation continued in Urbana and Texas.

A third substance, protogen, necessary for the growth of *Tetralymera geleii* under purification in the Lederle research laboratories, appeared to be a related material. A fourth substance emerged during the summer of 1949 while I was teaching in Berkeley. H. A. Barker was led to recognize the similarity of our factor with a *Butyribacterium rettgeri* factor that he and Kline found essential for growth of *Butyribacterium rettgeri* with lactate as carbon source. Its identity with POF was established by our exchange and analysis of samples and afforded the pleasure of a publication with Kline & Barker (77).

The oxidation of ketoacids (41, 42) via transfer of aldehyde to thiamin pyrophosphate with concomitant CO_2 release, followed by reductive acylation of the cyclic disulfide form of lipoate to form the secondary 6-thiol ester, affords a unique energy–conserving process (47). The reaction cycle is completed by transacylation to coenzyme A and regeneration of the lipoyl disulfide via a flavoprotein catalyzed pyridine-nucleotide reduction. This reaction sequence, like the pyridoxal-phosphate role in amino acid metabolism, is now standard textbook biochemistry (42, 46, 101a).

5 SEARCH: CHOICE OR CHANCE

A move to Indiana University in the fall of 1947 brought adequate housing for my growing family. With a new pair of twins, a change in work environment, and a style of living that was hardly dull, the stress of leaving upstate New York after 15 pleasant years was nevertheless considerable. A group of excellent students soon developed, and, in parallel, a stimulating interaction with the talented virologists-geneticists surrounding S. E. Luria, (Lu to me, Salva to many). A warm, stimulating, and lasting friendship developed, following his initiative during my first visit to consider the Indiana post (for Luria's view, see 81b). Access became open also to the Delbruck-Hershey-Luria International Midwestern Phage Meetings, including among the attendees Renato Dulbecco, Josh Lederberg, Aaron Novick, Leo Szilard, and Jim Watson. Choice was quickly increased by seminars, lectures, and conversations with the genetic pioneers. More reflection developed in the physics-genetics work mode of more formal hypotheses, models, and planning that benefited our work with the "dirty chemicals." The Indiana scene was mutually strengthened to provide enlightening opportunity to each bright mind for exploitation' according to individual design.

The Hoosier legend was epitomized by the clarity, intelligence, wisdom, and modesty of Fernandus Payne, former graduate dean, who had gathered, nurtured, and guided the superb biological strength at Indiana University. His interaction with Herman Wells and Herman Briscol, the president and provost, defined with the faculty an atmosphere of creative availability without recourse to bureaucratic procedures. This allowed smooth movement to solutions of "simple" problems before they became major. The standards of friendliness, excellence, and acceptance of Tracy Sonneborn, Hermann Muller, and the biochemists Felix Haurowitz and Harry Day, fulfilled one's concept of a university. Off-campus invitations resulting from the pyridoxal phosphate–amino acid and ketoacid-lipoic successes fostered a recognition by colleagues new to me and provided additional education. Three years of accelerated learning and contribution, with opportunities to travel, lecture, and study, brought also proposals to move again. An offer of a teaching/research post, to build with initiative and freedom as a participant in forming a full-quality department at Illinois was accepted for the fall of 1950.

Between Indiana and Illinois a Guggenheim fellowship afforded a sojourn, which I term "Severo, Seymour, and Alice," with Severo Ochoa in the old Pharmacology Laboratory of New York University Medical School. It contributed greatly to my personal and professional education and growth. When I arrived in early February, Severo proposed that I join Seymour Korkes, assisted by Alice Del Campillo, on pyruvate oxidation studies with *E. coli*, contemplated to yield the necessary "active" intermediate for citrate generation. Severo said that the laboratory and the desk he had intended for my use

had become occupied; he suggested that because he was quiet and occupied his office only for short periods, I might find convenient the use of a spare table there. His hospitality and kindness proved extremely agreeable. Even when Irma arrived to take dictation and I rose to leave, Severo cautioned that he spoke quietly, would take but a moment, and felt I would not be disturbed if I continued to work. A stress-free, nontaxing environment quickly reoriented my personal emotions and thinking. My prejudices from earlier studies of pyruvate metabolism with lactic bacteria were tolerated and modified. I became enmeshed in a hospitable laboratory with Seymour and Alice. We formed a lasting bond as a working family of diverse aims, skills, backgrounds, and temperaments.

In midsummer, Severo and Carmen departed for Spain, and Seymour left for a windjammer holiday in the Caribbean. Alice and I remained to complete the experiments, working until the last possible moment. I went to Ithaca for a few days of sailing, and I assembled the data, which Carol typed into tables and sent with a letter to greet the return of Severo and Seymour to New York. There they cast the paper for the *Journal of Biological Chemistry* (78). Such experiences are as indelible as infrequent.

My 1950 move to Illinois brought me into close contact with superb chemists who had attitudes akin to the biologists at Indiana. The Illinois plain was nearly bare of microbiology when George Stoddard arrived as president. Two earlier ages of magnificence had passed. T. J. Burrill taught the first course in plant bacteriology in America in the 1880s, before assuming the university presidency, and Otto Rahn built a quality department again in the 1910s, but it drained away as World War I began. The third rebuilding, in the 1950s, gained a helpful prod from E. B. Fred, the Stoddard counterpart at the University of Wisconsin. He engendered resolve and imagination, and George Stoddard had the resources to ensure a carry-through. When Luria and I arrived, Orin Halvorson and Sol Spiegelman had already begun work at Illinois. The chance to build a complementary education in general microbiology, with chemistry, virology, and genetics, was soon realized. The goal was for each to offer a lecture and a laboratory course at the current state of the art in the area of primary interest and expertise. Based on our experience at Indiana, Luria and I jointly introduced a general biology course for the junior-graduate level that focused on the structure and function of microbes. The impetus for the course came from my Cornell experience and drive and our mutual Indiana teaching blend; it began as three fourths my responsibility but the full course tapered to Luria until I moved to biochemistry, when he assumed the full course. His strength soon developed and with it the courage to carry the *Journal of Biological Chemistry* from my bookshelves to his without a brown paper wrapper.

My arrival in Urbana—from the Finger Lakes, via New York City and the limestone hills of southern Indiana, and despite my early flatland rearing and

the richer soil of Illinois—was indeed a shock. The rivers, lakes, and parks of Champaign County—Crystal Lake and Lake of the Woods—could hardly replace Cayuga, where I had a cottage, a sailboat, and a gorge. I missed the shaded trails from my home to the laboratory, the polo horses to ride in the country, and in New York the Saturday noons in the Ochoa laboratory followed by an afternoon ballet, dinner, or an evening play—let alone the finely equipped and staffed laboratories and the easygoing, "as you wish" discourse with colleagues. Urbana was hot, humid, and depressing: a 9' by 22' converted library, without air conditioning, next door to an unfinished laboratory. The tempo was soon obscured by the challenge of mountains of work and a wager with Luria on who could first initiate and complete an experiment. Encouraged by our own drive and the Stoddard follow-through, "You were brought to do a task and the requisites will be available," quality soon developed beyond that imagined by some fellow faculty.

Add to these the warmth, welcome, and support of the organic chemistry group around C. S. "Speed" Marvel, which was soon revealed by his invitation to me to join the organic-division faculty's twice-daily coffee consulting table. There my aims, plans, and needs were introduced, considered, and responded to. A comfort and regard developed. Dr. Fuson soon appeared in my ex-library to propose an invitation for an organic graduate student to join my laboratory to help with the pyruvate factor; soon, a second Fuson student joined our group. The lipoic story was completed, recorded, and summarized.

Similar welcome came from the biochemists, who provided a needed reagent or counsel freely, always accompanied by an offer of a quick talk over a cup at the local coffee shop. Our documentation of the ketoacid metabolic process and complexes (41) was extended by Lester Reed to the mammalian systems and later visualized by electron microscopy in spatial organization of the components (47, 101a).

The microbiology effort soon developed into an exciting network of learning for students and faculty. My Cornell experience in biology and chemistry, with introduction to modern genetics at Indiana, was extended by van Niellian choices from nature's warehouse and blended with Luria's virology and breadth of perception and Sol Spiegelman's superb genetic lectures. Orin Halvorson showed infinite patience to avoid, deflect, or dilute the sharp and intense exchanges eminating from the growth of the effort and the faculty. On occasion he harvested more rancor than was due his kindly, caring demeanor. We taught microbiology students and those in other areas of biology, and through the courses, seminars, and research forged interactions with the bio- and organic chemistry students and faculty that opened multidisciplinary efforts across the campus.

Within five years we had exhausted the wealth and facilities available to microbiology. A new building was authorized, planned, and constructed. I was

invited by lateral transfer to join the chemistry department, further the mix with biology, and design, staff, and lead the next generation of biochemical effort.

In September of 1955, after five years at Illinois, I began pointing biochemistry toward the final quarter of the twentieth century and beyond, after the 30-year chairmanship and the mountain of accomplishments of W. C. Rose. What seemed a reasonable five-year challenge required a decade of service in supplying the division with appropriate faculty, courses, and facilities while at the same time preparing in myself new skills in numerous areas of research and teaching. At the same time I dealt daily with the mix of faculty and student interests. Coping with the intense forces of a succcessful and solidly based department, I wrote fewer than 20 full papers in this decade. From the explorations started, however, new solutions continued to evolve in genetics, organismal growth, soluble enzymes, dynamics, and physical probes of structure.

The science developed in this decade, as amplified in the following four sections, include the terpene oxygenases, the mechanism of dioxygen reduction, and the entrance of biomolecular physics with modern equipment, methods, concepts, and theory. Within this period, the genetics of fluorescent pseudomonads emerged (see Section 6) with metabolic plasmids as potential extenders of their versatility in the biosphere. Both classical (see Section 6) and molecular (see Section 9) genetics were developed per se and to potentiate the physics, chemistry, and enzymology. The $P450_{CAM}$ (see Section 7) multienzyme system emerged as the model of choice for the important enzyme set of synthetic and detoxification monoxygenases. The introduction of physical probes by Beinert & Orme-Johnson at Madison defined the nonheme, redoxin, and P450 iron centers (see Section 7) that led directly to the building of biomolecular physics at Illinois with the groups of Hans Frauenfelder and Peter Debrunner (see Section 8).

Within this period also, Roger Stanier and I assembled and edited *The Bacteria*, a five-volume source book on the biology and properties of prokaryotes, with the premise of acquainting biochemists and physical scientists with the availability and power of the microbes to resolve general problems of living cells and organisms. The subtitles—Structure, Metabolism, Biosynthesis, Physiology of Growth, and Heredity—indicate the titular emphasis.

In university service, I was given a rare professional challenge. I was requested to build, next door to the new, excellent microbiology department, a biochemistry component of first magnitude in an outstanding department of chemistry. This came at a moment of the emergence of biology to chemical and physical probes and theory. Rebuilding a faculty was clearly indicated, owing to decreased access to two top stars because of retirement and reassignment and the rapid developments in scope of the biological sciences. Many of the pluses available to the task I have acknowledged previously (44, 45). In the 1955–65

decade, external funds were increased markedly by a revival in focus on science, with biology a major target. The Illinois biochemistry faculty was doubled, funding increased more than an order of magnitude, and teaching and research reached the national attention level. Facilities were added as required, and physical methodologies were inserted and polished as animal and microbial cells became tools of inquiry. Top-flight faculty and visitors were recruited from the leading education centers of the US and abroad. New mixes developed with the arrivals: from physics, E. S. Lennox; from biochemistry/biophysics, Gregorio Weber; from chemistry/medicine, George Schroepfer; from biology, John Clark, Woodland Hastings, and William Rutter; in proteins and carbohydrate chemistry, Finn Wold and Bob Barker; and in intermediates, Lowell Hager and John Wood.

Access to the U. K. faculties of Sheffield and Leeds enriched our growth in teaching and research. An exchange program with the Sheffield University Biochemistry Department brought to Urbana, for a year or longer, Gregorio Weber, Quentin and Jane Gibson, Vincent Massey, and John Sykes, while Hastings and Wold gained a year of English professional experience. The Dagley school from Leeds brought us first Peter Chapman and Peter Trudgill, then Dave Gibson (microbiology) and John Wood, all of whom added superbly to our understanding of the blending of clean chemistry and imaginative biology. The mentor Stanley Dagley became our first N. S. F. foreign visiting professor to meld science, culture, spice, humor, and a mature, yet always new, view of biology and chemistry. He even made the first known creative use of "Boneyard Creek," the drainage ditch running through campus, by sampling its rich and varied enrichment cultures from his overhanging porch.

6 PSEUDOMONAS GENES AND METABOLIC PLASMIDS

We gathered a set of questions under the concept of the genetics of anticipation. Metabolic diversity in the biological recycling of organic structures, both natural and synthetic, led us to explore the organization, expression, and selectivity of genes for catabolic pathways. We wondered how the limited amount of DNA per prokaryote chromosome imparted the nutritional diversity ubiquitously distributed among aerobic saprophytic strains. These concerns were augmented by the need for biological responses to the synthetic powers of industrial chemistry. We viewed it as a problem of evolving populations and of exchange mechanisms among individuals; we formed questions reducible to a working hypothesis and to experimental approach. If populations compose the information base, gene exchange must ensure the maintenance of the repositories made visible under continuing and identifiable selection pressures. Questions of gene transfer among homogeneous vs heterogeneous strains, species,

and genera emerged; the counterthought was the maintenance of the species and the survival of recombinants. The role of ecological niches (e.g. the *E. coli* strains of the intestines or methanogens in the stagnant organic residues of eutrophic ponds or sewers) raised the possibility that additional biological factors that maintain characters attributed to species and genera (83a).

We were led to the fluorescent pseudomonads by several features: the visual choice of clones from enrichments; the quality and abundance of chemical transformation data from the Delft, Dagley (Leeds), and Evans (Bangor) schools; the ease of cell breakage; the Stanier-Doudoroff-Palleroni "opus" (101); consecutive summers with van Niel and Stanier in Pacific Grove and Berkeley; and the popular little book of Otto Rahn, proclaiming the "amazing appetites" (94). The related aerobic strains, with free-living "nitrogen fixation" power—many amply able to invade plant tissues and fix nitrogen or destroy tissue (i.e. "plant pathogens")—were an added bonus. The working concepts of the genetics of anticipation included bacteriophage as possible repositories of information and fertility for gene transfer. The persistence in nature of phage DNA packages with a protein coating seemed a reasonable adjunct to the well-established survival potential of soil and water saprophytes under limited nutritive conditions and changing environments.

We approached the diversity of metabolic patterns by selecting organisms with pathways previously unexplored with respect to intermediaters, enzymes, mechanisms, and catalytic centers. We decided to invade the poverty of knowledge on hydrocarbon metabolism and oxygen-incorporating mechanisms coupled with the problems extant from the limited solubility of aliphatic, aromatic, and terpene (steroid) hydrocarbons in the aqueous milieu customarily employed in biological experimentation. We sought classes of compounds offered by nature in broad structural diversity, keeping alert to possible patterns of metabolic convergence as a clue to reducing the total genetic load. The boundless variety and abundance in plant residues of isoprenoid compounds presents a diverse source of growth substrates to microorganisms. Additional problems were raised by mammalian and fungal biologists as they attempted to understand the chemistry and enzymology of isoprenoid intermediates in steroid biogenesis and in the formation of several types of derivatives of major regulatory import, particularly "hormones" and "bile acids." The environment in Urbana, with constant stimulation, interaction, and access to excellent organic chemists [e.g. their input to the POF (lipoic acid) problem] led us to discuss, then explore, the vast array of hydrocarbons for biological turnover. Mevalonate, the precurser of terpenes as an intervention between acetate and squalene in cholesterol biogenesis and the array of phosphate derivatives afforded a clue to the solubility problem.

These problems did not arrive in our thoughts fullblown. Primitive early inklings arose in conversations at Cornell with William Mansfield Clark and

Barnett Cohen during their presentations of seminars and our preparation of their bulk quantities of organisms. They were further developed in our evening musings about what man would next make that nature could not handle: the derived products, the cellophane wrappers, the lignin in the West Virginia streams as a result of polycellulose films and fiber, the white vs black rot of stumps in postforest terrain. Such items were sorted at the organic chemistry coffee table as I explored in Karrer's textbook the array of potential target organic compounds for mechanisms of biological attack. E. J. Corey, with interests in mono- or sesquiterpenes, offered to determine the organic structures if we would extend the biological aspects (i.e. accumulate the intermediates and develop and solve the enzymology and the enzyme mechanisms). The aromatic series was considered with the organic chemists Will Gruber and his wife Edna, who joined our group with Dr. Fuson's stimulus. Edna's syntheses gave us a fine avenue to the ortho-meta fission of aromatic rings, following Stanier's observations on mandelate enzymology and the chemistry and biology of aromatic fission elaborated by the Dagley School.

Will Bradshaw joined us after completing his Ph.D. in the H. A. Barker laboratory; Robert Baum entered as a graduate student from the Cornell microbiology unit. Enrichments with terpenoids (Will primarily with the bicyclic and Baum with the monocyclic) provided organisms able to use a variety of terpenoids as sole carbon and energy sources; later, additional terpene-competent cells were furnished from the Bhattacharyya laboratory at the Indian Institute of Science in Bangalore (32). Bradshaw emerged with two cell types, each selected as able to grow on the bicyclic D-(+)-camphor, (2-bornanone): (*a*) a waxy, orange-pigmented strain, later assigned for Chapman as *Microbacterium rhodocorous* (8, 21, 52), and (*b*) several fluorescent pseudomonads (43), assigned by Stanier to the *Pseudomonas putida* species.

The chemistry of pathway and enzymes was developed by H. E. (Ed) Conrad, who, with Bradshaw and then E. J. Corey, identified the intermediates and constructed a potential pathway first in a short 1959 paper (13a). We searched for induction patterns, first in whole cells, and then turned to extracts, separating the enzymes, looking at mechanisms, and characterizing the new reaction centers and their reaction states. The first reaction obtained in extracts by Conrad was for a ketone-lactone conversion of D-camphor to the 1,2-lactonide, a new enzyme type we termed ketolactonase.

The ketolactonase I proved to be a multienzyme system, driven by NADH through an FMN-reductase (104) coupled to the substrate monoxygenase, an iron-flavine complex (105). A dozen papers defined the components, characterized the substrate range, and projected as ketolactonase II a similar enzyme system able to accomplish fission of the second carbocyclic ring (22–24, 53). Only recently Peter Trudgill, who characterized the FMN-reductase and complex of ketolactonase I, revisited the camphor enzymes to show that ketolacto-

nase II proceeds via the coenzyme A thiol ester with proton elimination (89, 102a).

Jens Hedegaard arrived from Denmark to solubilize and define the first methylene hydroxylase (70, 71), later shown to be P450 heme sulfur enzyme complex (59). Throughout Jens's work, Ed Conrad's data, advice, notebooks, and compounds were available as an "open book." The quantities of the critical intermediates required for this work were prepared in crystalline form from fermentation beer (spent-growth media) by two young ladies. These studies were summarized in 1965 at the opening of the Hadassah Medical School (54). The biological facets were added in honor of Kees van Niel's seventieth birthday (50). The chemical and enzymatic studies of the Conrad-Corey collaboration with the Bradshaw *Pseudomonas* were extended and presented with the Jacobson and Bertland analyses of the growth characteristics and the enzyme induction patterns. The phenotypes for genetic advances on the metabolic plasmid grew from these studies and from the extensive, never-published work by Rick Prairie on terpene alcohol dehydrogenase.

The comparative metabolism was constructed from a near-zero base, as were the equally essential genetic tools and organization. Alex Bertland came from Delaware, isolated phage, and characterized the properties: Chakrabarty extended the range of phage and initiated genetic exchange via transduction (16, 51) and then conjugation (17, 18). His phage, pf16, which was similar to the *E. coli* T-even phages in morphology and genome size (DNA ~ 100 daltons) although virulent, became the general transducing agent (19, 58). Niblack examined the morphology, serology, and DNA properties of about a dozen phages that had been isolated by Bertland and by Chakrabarty. The range was similar to the coliform phages.

This general approach calls to mind the first Max Delbruck contribution to biology—"Use a single bacterial culture, standardize conditions including media, and stop isolating phages." With *E. coli* strain B and seven phages, Delbruck dominated a cadre of coworkers and students who, with Luria, in part ushered in the age of molecular biology (14).

Conversations with C. Yanofsky, Phil Hartman, and Ed Adelberg convinced me that the tryptophan pathway could expedite a route to the genetic system(s) of the fluorescent pseudomonads. During Christmas break in 1965 with Irving Crawford in his laboratory, we characterized half a dozen tryptophan phenotypes (26). The following year, using Chakrabarty's methods, we accomplished by transduction with the pf16 system the construction of the three-operon gene map of the *P. putida* tryptophan synthetic pathway (51). A new control method for biosynthetic enzymes appeared, a welcome byproduct. When Crawford separated the *Pseudomonas* tryptophan biosynthesis enzymes by chromatography on sieve columns, a loss in anthranilate synthase activity suggested a two-gene, two-component array. Steve & Sherry Queener beautifully resolved these as a larger, chorismate-binding enzyme and a smaller, glutamine-sensing

moiety (93). Vicki Hewlett, an undergraduate hourly employee, responded in the summer of 1966 to our request to isolate *P. putida* auxotrophs via nitrosoguanidine, or methyl-methane sulfonate mutagenesis. She provided nothing in the first two weeks, one mutant in the third week, ten in the fourth week, and by the end of the summer, five hundred auxotrophic strains. When classified for amino acid or purine-pyrimidine requirement, the essentials for phenotypic scoring and genetic analyses were at hand.

On turning to the primary problem, camphor metabolism as a phenotypic representative of the diversity of substrate utilization, my wife Carol searched without success for transductional linkages of a variety of auxotrophic markers with camphor growth–defective loci. Later Helen Dunn did observe linkages of several auxotrophic loci to isobutyrate and acetate, the final intermediates in the pathway. Evidence forcing acceptance of the concept of an extrachromosomal (plasmid) array of the genes encoding enzymes for conversions from camphor to common, anaplerotic intermediates came from Jim Rheinwald. For his four-year bachelor's-master's thesis, Jim prepared and classified a library of camphor growth–defective strains and continued transductional mapping. Linkages were frequent among the "CAM" mutants and again they lacked linkages to the auxotrophic loci. Some mutations carried a group Cam^- phenotypic marker (i.e. failed to grow on any of the available pathway metabolites prior to isobutyrate). These were designated Seg, for segregation of the presumed "plasmid." A high-frequency preparation of Seg mutants by Chakrabarty with a mitomycin C treatment for "curing" permitted us to proceed with the concept of terpene metabolism encoded by extrachromosomal genes and to presume that other nonessential pathway genes (e.g. the aromatic series) may also be found on plasmids. Noel Dunn, a Bruce Holloway student from Monash University, accomplished the extension of the concept to the naphthalene plasmid (33).

Chakrabarty soon established a conjugation system for chromosome and plasmid mobilization. He used both defective phage and a CAM plasmid–associated fertility, thus establishing two sources of fertility for the *Pseudomonas* genus. Carol and I spent four summers (from 1963) with Al Chakrabarty and the Queeners at Cold Spring Harbor. We learned and polished the detailed genetics, consulted with the experts (particularly Al Hershey), occasionally worked at the bench with Luria—his son Dan was media cook—and brought Carol, myself, and a band of students to an acceptable mobile level for genetic analysis of the fluorescent pseudomonads. By the time Chakrabarty departed as an independent investigator and continuing collaborator, fluorescent pseudomonad genetics covered phage transduction, an extensive collection of chromosome and plasmid-borne mutants, and fertility factors.

The isolation of plasmid DNA had still eluded us when Jim Johnston asked to join our group, his preliminary data having suggested a route to metabolic

plasmid DNA. Over a two-year period of exploration, Jim reached suitable, repeatable methods for the isolation of the NAH, SAL, and TOL aromatic plasmids and an occasional success with CAM (74). Jim's DNA isolation methods permitted Roberta Farrell, a bright young graduate student, to compare the homologies of the DNA from aromatic plasmids encoding the TOL, SAL, and NAH pathway enzymes (34a, 35). Thus to our metabolic and classical microbial genetic armament, molecular genetics was introduced by Johnston and polished primarily by Farrell (35). As an effective extension to molecular genetic analysis, transposon mutagenesis, restriction-enzyme digestion, and cloning were added to analyze the organization, control, and expression in *Pseudomonas* and *E. coli* of the peripheral metabolic genes (See Section 9.)

7 THE $P450_{CAM}$ MODEL AND ACTIVE O_2

The P450 field has shown a prodigious growth over the past two decades in numbers of investigators and importance to modern biology, biochemistry, and physics (96). The P450 proteins fall into two ranges of regio- and stereoselectivity with micromolar and millimolar affinities (25a, 56) and are essential to biosynthetic and to detoxification processes. The $P450_{CAM}$ system initiated and developed in our laboratory has enjoyed a model role in defining two new catalytic sites, a carbon-and-dioxygen-substrate reactive heme sulfur center (46, 56) and the iron-sulfide redox-effector moiety (25, 27, 31, 106). These proteins are soluble, available in quantity, and form complexes of high activity and selectivity. The iron centers are excellent reporter groups for physical probes, examined in multidisciplinary studies with colleagues in the University of Illinois Physics Department. More recently we have developed genetic amplification, control, and modification and have broadened the array of systems subjectable to heterologous complementation in defining the domains in physical and catalytic parameters.

Our studies have developed through half a dozen phases, with a number of appropriately talented investigators in each. They may be characterized briefly as:

1. Isolation by enrichment on terpene hydrocarbons of an array of fluorescent pseudomonads (13a) subject to ease of production with high levels of the relevant enzymes, coupled with the ease of protein release and separation (64a, 119).
2. High-level production and rigorous characterization of the essential components. Pre- and posttranslational isotope replacement by growth and reconstitution have given definitive solutions with physical probes. Crystallization of the $P450_{CAM}$ has provided "pure" protein for primary (66, 67, 102) and tertiary (91, 92) structure analyses and for alternate (metallo) atom replacement (111, 112).

3. The application of physical probes (e.g. EPR and Mössbauer) to the iron-sulfide and the heme proteins. Later flash photolysis to the latter proved particularly valuable and led to development of biomolecular physics in the Frauenfelder-Debrunner groups on the Urbana campus, (see brief outline in Section 8).
4. Broad characterization of the heme and sulfur-iron states, the stable intermediates of a four-step reaction cycle, defined by two substrate additions and two single electron–transfer reactions (59, 69, 98). The potentiation by solute and protein interactions over multiple temperature and time domains emerged in part from optical and in part from other resonance probes (see section VIII).
5. Homologous domains of primary structures (13, 37a, 68, 116) among components differing in substrate selectivity. The tertiary folding of the protein sequences of the $P450_{CAM}$ defines the heme and substrate ligands and catalytically essential group interactions at a 2-Å level by X-ray diffraction.
6. A short (peroxy) reaction cycle of single oxygen donors to the ferric heme protein (C. Jung, et al; 63).

A field of oxygenation and the structure of "active oxygen" are defined for the heme sulfur active center (see 84, 96).

We have recently added two monoterpene heme sulfur P450 monoxygenase systems analogous in components and reactions to the $P450_{CAM}$ system. These permit measurement of affinity and dynamics of homologous vs heterologous subunit complementation to define the active site, substrate, and binding redox domains (108; J. Ullah, et al, manuscript in preparation).

Collaborators at each critical stage in the development of the $P450_{CAM}$ model are indicated in the appended references to primary and review publications. The multidisciplinary skills developed at Illinois between the physical and biological sciences during the past two decades are a major force in the advances of biomacromolecular methodologies, concepts, and planning, both nationally and internationally. Numerous fruitful collaborations have been fostered; new investigative skills and a new cadre of investigators have emerged.

8 PHYSICAL PROBES AND BIO-REPORTER GROUPS

Enzymes with new, important catalytic-reaction centers emerged from the pre-1960 cell isolates of Bradshaw (13a) and the substrate structure identifications of Corey with the discovery of two types of iron-containing monooxygenases (22–24): a ketolactonase by Conrad, and the first soluble methylene hydroxylase by Hedegaard (70, 71). Both are multicomponent systems driven by NADH energy via flavoprotein dehydrogenases. Each contains a "nonheme iron" center, and the hydroxylase a heme-sulfur substrate selective

center as well. The iron atom is an excellent reporter group to resonance probes of varying resolution in time, state, and sensitivity and permits definition of active site structure, the stable and transient intermediates, and the reaction cycle.

The ketolactonase system contains iron, with flavine, in the substrate-selective and dioxygen-reactive protein of currently unknown ligand structure and reaction sequences (24, 105). Taylor & Trudgill have restudied the ketolactonase component substrate reactive component, which they have identified as a single FMN-bound 78-kD enzyme composed of two subunits of equal molecular weight (102a). In contrast, the hydroxylase-iron centers have been extensively characterized as a redox-effector of the two-iron, two-sulfide acid labile class denoted as $Fe_2S^*_2Cys_4$ class (81a, 97) and a protoheme with cysteinyl axial ligand that demarks the reactive hydrocarbon oxygenases of P450 proteins (28, 61, 79, 99).

Physical measurements on the hydroxylase components began with our pure redoxin; Helmut Beinert experienced a full $g = 1.94$ EPR spectrum, attributed to the nonheme iron center (31). Mössbauer spectra by Roger Cooke of the Frauenfelder-Debrunner laboratory in the University of Illinois Physics Department followed as we prepared the iron-57–substituted molecule (25). An active collaboration thus began that has grown over the past 15 years. The elegance of the physics input has advanced Illinois to a unique center in measurement, theory, and variety of probes available. From the biological side, our contribution of a P450 system as the model of choice has been extended in variety of inquiry and, more recently, in related systems (see discussion in Section 7). In the present context the growth and evolution of this multidisciplinary approach to biomacromolecules and active centers can best be realized from the publications and reviews in biological and physical compendia.

The physical measurements require pure enzymes of authentic structure. For the redoxin these came from David Cushman (27), Randolph Tsai (107), and detailed technical advances by John Tsibris (88), particularly with stable isotope substitutions of iron, sulfur, and other chalcogenides. The advances of the $P450_{CAM}$ arose from Professor Katagiri's (75) preparation in pure form of the protein after separation from the redox flavoprotein. Chang-an Yu prepared the first crystalline P450 (119) and John Lipscomb and Stephen Sligar (initially a physics graduate student with Debrunner) provided broad and definitive spectroscopic understanding, both dynamic and equilibrium (84). Gerald Wagner prepared a tetragonal crystalline form and a second orthorhombic crystal form suitable for X-ray analysis and for broad variety of single crystal polarized optical (29) and EPR (30) measurements. His sulfur and iron replacements defined in the Champion resonance Raman measurements (20) the heme iron with thiol axial ligand that characterizes the P450 species. Single-oxygen-donor chemical probes extend the nature and reactions of the active center in the hydroxylation process (63, 110).

A variety of collaborations has arisen with the high purity supply of protein available in quantity to investigators in addition to the Frauenfelder-Debrunner group and former students. Outstanding feedback has come from Eckard-Münck, Paul Champion, Stephen Sligar, and others, especially the Pierre Douzou laboratories in Paris and Montpellier, who have added low-temperature and cosolvent technology and interpretation (32a, 103).

The three-dimensional structure of $P450_{CAM}$ has been determined by the Kraut group at the University of California, San Diego, with the orthorhombic II crystalline form prepared in our laboratory by Wagner. The solution was completed by the primary structure of 412 residues recently obtained by the Yasunobu group on the Manoa campus in Hawaii (66–68).

Early advances in understanding the reaction rates in formation of ferrous carboxy adducts to P450 at low temperature arose from observations of Vincent Marshall and were extended and theoretical interpretation provided by molecular studies of the Frauenfelder group in physics (4, 6). Refinements in dynamic analyses of the structure and function of myoglobin and the subunits of hemoglobin over hitherto unavailable temperature and time ranges have resulted from their advances in equipment design to accomodate low as well as ambient temperature, shorter reaction times, and conceptual advances in models, observations, and data transformation, interpretation, and presentation (2–5). The original flash photolysis paper of the Frauenfelder group (6) on P450 advanced into a now detailed presentation and theoretical interpretation in two papers on myoglobin in biochemistry (3, 5). Their effort also found tunneling kinetics in biomacromolecules at low temperatures (1), and with Petsko analyzed the motion of proteins in crystals as a temperature function (37). Current advances in reaction theory have also appeared (10).

The Debrunner Mössbauer effort has expanded into single-crystal EPR and polarized optical measurements, in primary collaboration with Gerald Wagner from our laboratory. The advances in teaching and methodology have now infused other efforts of the biochemistry department, including chloroperoxidase, the P450-like protein, under long term study by the Hager group, and the Fe_4S_4 center concerned in protein turnover (109), under study by the Switzer group.

Another collaboration initiated with Pierre Douzou and Pascal Debey at Edmond Rothschild Institute (later the Museum of Natural History in Paris) has broadened the temperature and cosolvent range to first characterize, with Laura Eisenstein, the labile ferrous oxy substrate state of $P450_{CAM}$ (33a) and later the velocity of the more transient individual reactions of the hydroxylation cycle (81) by low temperature stop-flow spectroscopy (80, 81).

Inadequate as this description is in visualizing the enormous value of these physical probe analyses of biomacromolecules, the area continues to be one of sustained excitement, potential, and growth. As pointed out by Professor Frauenfelder, the success of contributions to science can be measured in

several ways. First advances may become standard and lasting textbook presentations; or, lacking this, find their way into important reviews, documenting the state-of-the-art in the field. A third level citation is for the conceptual and factual value in manuscripts of original publications. Beyond this the rewards are few.

9 MOLECULAR ORGANIZATION FOR REGULATION OF DIVERSITY

Molecular genetics—the opportunity to examine gene structure and control in metabolic plasmids—began with I. P. Crawford in a year-end work holiday, about ten years after our first scoring of the locus and organization of the tryptophan biosynthetic genes in *Pseudomonas*. Crude DNA samples of two plasmids, pDG7 and pAC1, encoding the naphthalene and the salicylate oxidative pathways, were supplied by Jim Johnston. When subjected to restriction enzyme digestion and agarose gel electrophoresis, the fragments were similar in number and size, with the exception of one slightly larger segment from pAC1 (35). Roberta Farrell extended the quality of plasmid DNA recovery and found annealing homology of all respective restriction fragments from pDG7 and pAC1. She also found about 70% homology between these fragments and fragments from the strain mt2 (34, 34a).

Following 1975, advances by Johnston (74) gave us a routine capacity to isolate the aromatic plasmids of about 100 kilobases. But unfortunately we found only scattered, infrequent success with the terpene plasmid, CAM, of $\geq$ 200 kilobases. Hideo Koga engaged one exception in the isolation from a gene library, the CAM plasmid, pRG1, the clone of the *camC* and *D* genes. These genes encode respectively $P450_{CAM}$ heme protein and the 5-*exo* alcohol dehydrogenase. We shall return to the Koga experiments in a moment.

Our primary development of methods for analyses of *Pseudomonas* plasmid expression and control has used the naphthalene plasmid, pDG7, with lesser emphasis on the related salicylate phenotype of plasmid pAC1 isolated by Chakrabarty, and the TOL plasmid from *P. putida* strain mt2. The latter has received extensive analyses in the laboratories of Williams (115), Timmis & Bagdasarian (7, 36), and Nakazawa (73). The commonality of chemical steps, as detailed by the Dagley, Evans, and Stanier groups, are reflected at the genetic level for catechol fission by the plasmid-encoded aromatic ring *meta* pathway enzymes and the chromosome-encoded *ortho* fission enzymes (33), as first demonstrated by Dunn (38, 65, 118), and extended by the molecular genetic analysis of the plasmid DNA.

Significant contributions from our laboratory by Yen, Grund, & Koga will be reviewed briefly. Yen, a talented lambda geneticist with a Ph.D. from Iowa with Gussin, joined our group with an aim to learn biochemistry and enter the *Pseudomonas* plasmid area. He prepared Tn5 insertion mutants of the

pDG7 plasmid, classified the lesions by enzymatic assay, and prepared a restriction map (65, 117). Later, with Marjorie Sullivan, a heteroduplex electron microscope analysis of the DNA fragments (118) also revealed the fine structure differences of the related salicylate plasmid, pAC1, in the *nahA* locus.

In the plasmid, pDG7, the naphthalene pathway enzymes are encoded in two gene clusters under pleiotropic control. The first operon controls the enzymes that convert naphthalene to salicylate and the second controls the reaction from salicylate via catechol to the anaplerotic intermediates, aldehyde and pyruvate. These operons of $\sim$ 10–15 kilobases are separated by about 3–5-kilobase segments that carry a $\leq$ 1-kb regulatory locus, *R*. The salicylate plasmid pAC1 has undergone a deletion and received an insertion in the *nahA* region to leave unexpressed the coordinately regulated gene of the *nahA-F* operon.

Alan Grund showed *trans* dominance of gene complementation and expression by cloning with compatible vectors (57). The regulatory gene, *nahR*, with unexpressed structural genes in *trans* array, gave complementation indicative of positive control (38). Yen has employed two compatible vectors with the Grund pAG15 clone, carrying in vector pRK290 the R1 fragment encoding genes *nahA-K*, and in vector pKT240, the *nahR* gene.

A few words are in order concerning the Koga success in cloning the *camC* gene in vectors pKT240 and pBR322. The former is expressed both in *P. putida* and *E. coli* at similar constitutive levels, each about a five-fold induction of enzyme expression in the presence of the inducer D-camphor. Promoter/operator expression from the pKT240 vector occurs by different polarity of readthrough in *Pseudomonas* and *E. coli*. In *E. coli* the direction correlates with that expected from the ampicillin promoter, in *Pseudomonas* from an unidentified region upstream to the BstEII site. In *E. coli* the vector pBR322 locus expression of *camC* again is as expected for the Ap^r promoter.

Comparative studies of the CAM P450 system with additional monoterpene P450 systems associated with metabolism of the acyclic compound linalool and the monocyclic compound para-cymene became possible with the availability of strains from P. K. Bhattacharyya of the Indian Institute of Science in Bangalore (32). The P450 proteins, in common with the camphor system, are highly selective, of high affinity ($\sim 10^{-6}$ M for homologous substrates), and carry specific flavin and redoxin enzymes with physical properties similar to the $P450_{CAM}$. Linalool is hydroxylated on the 8-methyl group; 70+ analogues have been prepared to map the substrate site. A third system, para-cymene, is effective with the hydroxylation of the methyl alkyl group. The linalool system components show limited cross activity with the Cam enzymes whereas the para-cymene, heme sulfur component, $p450_{CAM}$, is more reactive. In heterologous association, similar protein-protein affinities, but reduced redox-effector active capacity, was noted.

These starts in the molecular genetics area now have sharpened tools to allow

analysis of the structure and organization of the three proteins of the $P450_{CAM}$ system. The nature of the induction and the similarity among the aromatic plasmids also can be analyzed. Much has been done; intriguingly, much remains.

10 AND

This essay of learning, personal in reflection, rekindles feedback from colleagues, classes, and laboratories and a breakthrough in the first post-Ph.D. decade. Fired by a beckoning of the bright, unclouded future of pre-World War II, as doubts faded perceptibly, the second decade brought equally a new departure. We acquired an introduction to multicomponent catalytic complexes and a new chemical that was functional in a unique biological reaction of energy capture and delivery.

A growing acceptance in the 1950s brought superb biology with chemistry among open, talented, imaginative—if competitive—colleagues. Motivated, able students and friends emerged to wander free among the growing facts, then to quicken with staccato steps along a charted path. And so with this exposé, can personal memory—distorted with bias to enhance, diminish, or discard—still distinguish chance from choice? History seeks toward, but is not, truth.

Personal growth was fostered amid familial support, tension, innovation, and reaches for excellence by those who dared. Searches were abundant for self, for students and colleagues, with the price of failure seldom so high as to quench the pursuit.

Genes appeared as chemicals codified with the transforming principle of the pneumococcus in the superb multidisciplinary assemblage of "The Professor" (32b); the chemistry followed from Rollin Hotchkiss (14, p. 180). Separate evidence emanated from the incisive Hershey-Chase phage injection lacking sulfur protein (14, p. 100). Mutation and selection, as chance and choice from Luria and Delbruck (14, p. 173), and phage recombination brought in abundant variety the accumulating evidence and motivation to delve into the mysteries.

Soon we asked how the pseudomonads anticipate the variety in organic structures synthesized by man and nature. The questioning directed us to repositories of genetic diversity in the form of plasmids and their participation in extrachromosomal exchanges. The memory and action, of genes and enzymes, control the selectivity of convergent and divergent catabolic potential.

Biology provided systems to biochemistry for separation and molecular definition; chemistry defined the reactions centers, their substrates, and their modification, and physics defined the states. As physics entered, time domains were shortened, temperature ranges expanded, and with cosolvents multiple transient and static intermediates were delineated in the catalytic cycles of the active centers. New iron structures were identified, and their role in production of "active" oxygen defined.

Multidisciplinary effort refined the precision and the formulation of models; innovations were introduced in sources, detectors, data collection, and transformation. Soon dissertations evolved from descriptions of intuition toward models of action. Does one couple the principles of selectivity and molecular regulation to structure or to the maintenance of diversity in a dynamic spiral? Does change become the only constant between the hunches of intuition and the formulation, measurement, and conclusions toward a common thread of general meaning? Some "facts" are still too recent to judge their impact.

Truth remains a common goal, and the central dimension of "a university." In a modern Harryism (H. G. Drickamer), "Competence is the enemy of excellence"; or, from James Baldwin to his nephew, a century after Emancipation (7a): "You were not expected to aspire to excellence, you were expected to make peace with mediocrity." Baldwin also wrote: "Take no one's word for anything, including mine—but trust your experience. Know whence you came. If you know whence you came, there is really no limit to where you can go." And, from Carl Becker (9a), "How new will the better world be?"

Literature Cited

1. Alben, J. O., Beece, D., Bowne, S. F., Eisenstein, L., Frauenfelder, H., et al. 1980. *Phys. Rev. Lett.* 44:1157–60
2. Alberding, N., Austin, R. H., Chan, S. S., Eisenstein, L., Frauenfelder, H., et al. 1978. *Biophys. J.* 1001:319–34
3. Alberding, N., Chan, S. S., Eisenstein, L., Frauenfelder, H., Good, D., et al. 1978. *Biochemistry* 17:43–51
4. Austin, R. H., Beeson, K., Eisenstein, L., Frauenfelder, H., Gunsalus, I. C., Marshall, V. P. 1974. *Phys. Rev. Lett.* 32:403–5
5. Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., Gunsalus, I. C. 1975. *Biochemistry* 14:5355–73
6. Austin, R., Beeson, K., Eisenstein, L., Frauenfelder, H., Gunsalus, I. C., Marshall, V. P. 1973. *Science* 181:541–43
7. Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B., Bagdasarian, M. 1983. *Gene* 26:273–82

7a. Baldwin, J. 1963. *The Fire Next Time*. New York: The Dial Press, 120 pp.

8. Baum, R. H., Gunsalus, I. C. 1962. *Bacteriol. Proc.* 31 (Abstr.)
9. Bazin, M., Pierre, J., Debey, P., Santus, R. 1982. *Eur. J. Biochem.* 124:539–44

9a. Becker, C. L. 1943. *Cornell University: Founders and the Founding*. Ithaca: Cornell Univ. Press. 240 pp.

10. Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., et al. 1980. *Biochemistry* 19:5147–57
11. Bellamy, W. D., Gunsalus, I. C. 1944. *J. Bacteriol.* 48:191–99
12. Bellamy, W. D., Gunsalus, I. C. 1945. *J. Bacteriol.* 50:95–103
13. Black, S. D., Tarr, G. E., Coon, M. J. 1982. *J. Biol. Chem.* 257:14616–19

13a. Bradshaw, W. H., Conrad, H. E., Corey, E. J., Gunsalus, I. C., Lednicer, D. 1959. *J. Am. Chem. Soc.* 81:5507

14. Cairns, J., Stent, G. S., Watson, J. D., eds. 1966. *Phage and the Origins of Molecular Biology*. New York: Cold Spring Harbor Lab. Quant. Biol. 340 pp.
15. Campbell, J. J. R., Bellamy, W. D., Gunsalus, I. C. 1943. *J. Bacteriol.* 46:573 (Abstr.)
16. Chakrabarty, A. M., Gunsalus, C. F., Gunsalus, I. C. 1968. *Proc. Natl. Acad. Sci. USA* 60:168–75
17. Chakrabarty, A. M. Gunsalus, I. C. 1968. *53rd Meet. Soc. Gen. Microbiol.*, pp. 12–13 (Abstr.). Edinburgh: Soc. Gen. Microbiol.
18. Chakrabarty, A. M., Gunsalus, I. C. 1969. *Proc. Natl. Acad. Sci. USA* 64: 1217–23
19. Chakrabarty, A. M., Gunsalus, I. C. 1969. *Virology* 38:92–104
20. Champion, P. M., Stallard, B. R., Wagner, G. C., Gunsalus, I. C. 1982. *J. Am. Chem. Soc.* 104:5469–72
21. Chapman, P. J., Meerman, G., Gunsalus, I. C. 1965. *Biochem. Biophys. Res. Commun.* 20:104–8
22. Conrad, H. E., DuBus, R., Gunsalus, I. C. 1961. *Biochem. Biophys. Res. Commun.* 6:293–97
23. Conrad, H. E., DuBus, R., Namtvedt,

M. J., Gunsalus, I. C. 1965. *J. Biol. Chem.* 240:495–503
24. Conrad, H. E., Lieb, K., Gunsalus, I. C. 1965. *J. Biol. Chem.* 240:4029–37
25. Cooke, R., Tsibris, J. C. M., Debrunner, P., Tsai, R. L., Gunsalus, I. C., Frauenfelder, H. 1968. *Proc. Natl. Acad. Sci. USA* 59:1045–52
25a. Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., et al. 1980. *Microsomes, Drug Oxidations, and Chemical Carcinogenesis,* Vol. I. New York: Academic. 618 pp.
26. Crawford, I. P., Gunsalus, I. C. 1966. *Proc. Natl. Acad. Sci. USA* 56:717–24
27. Cushman, D. W., Tsai, R. L., Gunsalus, I. C. 1967. *Biochem. Biophys. Res. Commun.* 26:577–83
28. Debrunner, P. G., Gunsalus, I. C., Sligar, S. G., Wagner, G. C. 1978. In *Metal Ions in Biological Systems,* ed. H. Sigel, pp. 241–75. New York: Dekker
29. Devaney, P., Wagner, G. C., Debrunner, P. G. 1979. *Bull. Am. Phys. Soc.* 24:319
30. Devaney, P., Wagner, G. C., Debrunner, P. G., Gunsalus, I. C. 1980. *Fed. Proc.* 39:1824
31. DerVartanian, D. V., Orme-Johnson, W. H., Hansen, R. E., Beinert, H., Tsai, R. L., et al. 1967. *Biochem. Biophys. Res. Commun.* 26:569–76
32. Devi, J. R., Bhat, S. G., Bhattacharyya, P. K. 1978. *Indian J. Biochem. Biophys.* 15:323–27
32a. Douzou, P. 1977. *Cryobiochemistry: an introduction.* New York: Academic
32b. Dubos, R. J. 1976. *The Professor, The Institute and DNA.* New York: Rockefeller Univ. Press. 238 pp.
33. Dunn, N. W., Gunsalus, I. C. 1973. *J. Bacterol.* 114:974–79
33a. Eisenstein, L., Debey, P., Douzou, P. 1977. *Biochem. Biophys. Res. Commun.* 177:1377–83
34. Farrell, R. L. 1980. *Molecular and genetic relationships of aromatic metabolic plasmids in pseudomonads.* Ph.D. thesis. Univ. Ill. Urbana. 134 pp.
34a. Farrell, R., Chakrabarty, A. M. 1979. In *Plasmids of Medical, Environmental and Commercial Importance,* eds. K. N. Timmis, A. Pühler, pp. 97–109. Amsterdam: Elsevier
35. Farrell, R., Gunsalus, I. C., Crawford, I. P., Johnston, J. B., Ito, J. 1978. *Biochem. Biophys. Res. Commun.* 82: 411–16
36. Franklin, F. C. H., Bagdasarian, M., Bagdasarian, M. M., Timmis, K. N. 1981. *Proc. Natl. Acad. Sci. USA* 78: 7458–62
37. Frauenfelder, H., Petsko, G. A., Tsernoglou, D. 1979. *Nature* 280:558–63
37a. Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K. Muramatsu, M. 1982. *Proc. Natl. Acad. Sci. USA* 79:2793–97
38. Grund, A. D., Gunsalus, I. C. 1983. *J. Bacteriol.* 156:89–94
39. Gunsalus, I. C. 1947. *J. Bacteriol.* 54: 239–44
40. Gunsalus, I. C. 1950. *Harvey Lect., Ser. XLV, pp.* 40–63. Springfield, IL: Charles C. Thomas
41. Gunsalus, I. C. 1954. In *The Mechanisms of Enzyme Action,* ed. W. D. McElroy, H. B. Glass, pp. 545–80. Baltimore: Johns Hopkins
42. Gunsalus, I. C. 1954. *Fed. Proc.* 13: 715–22
43. Gunsalus, I. C. 1958. *Symp. on Water-Soluble Vitamins; Int. Symp. on Enzyme Chemistry, Kyoto, Japan, Oct. 1957. J. Vitamin.* 4:1–3
44. Gunsalus, I. C. 1975. Int. Symp. on Enzymatic Mechanisms in Biosynthesis and Cell Functions, Barcelona/Madrid, Spain, Sept. 23–27, 1975. *Homenaje al Professor Severo Ochoa en su 70 Aniversario International,* pp. 144–45 (Abstr. 17)
45. Gunsalus, I. C. 1976. In *Reflections on Biochemistry, in honor of Severo Ochoa,* ed. A. Kornberg, B. L. Horecker, L. Cornudella, J. Oro, pp. 125–35. New York: Pergamon
46. Gunsalus, I. C. 1968. *Hoppe-Seyler's, Z. Physiol. Chem.* 349:1610–13
47. Gunsalus, I. C., Barton, L. S., Gruber, W. 1956. *J. Am. Chem. Soc.* 78:1763–66
48. Gunsalus, I. C., Bellamy, W. D. 1944. *J. Biol. Chem.* 155:357–58
49. Gunsalus, I. C., Bellamy, W. D., Umbreit, W. W. 1944. *J. Biol. Chem.* 155:685–86
50. Gunsalus, I. C., Bertland, A. U. II, Jacobson, L. A. 1967. *Arch. Mikrobiol.* 59:113–22
51. Gunsalus, I. C., Chakrabarty, A. M., Gunsalus, C. F., Crawford, I. P. 1967. *Science* 156:538 (Abstr.)
52. Gunsalus, I. C., Chapman, P. J., Kuo, J. F. 1965. *Biochem. Biophys. Res. Commun.* 18:924–31
53. Gunsalus, I. C., Conrad, H. E., Trudgill, P. W. 1965. In *Oxidases and Related Redox Systems,* ed. T. E. King, H. S. Mason, M. Morrison, 1:417–47. New York: Wiley
54. Gunsalus, I. C., Conrad, H. E., Trudgill, P. W., Jacobson, L. A. 1965. *Israel J. Med. Sci.* 1:1099–119
55. Gunsalus, I. C., Dolin, M. I., Struglia, L. 1952. *J. Biol. Chem.* 194:849–57

56. Gunsalus, I. C., Ganguli, B. N., Katagiri, M., Tsibris, J. C. M., Debrunner, P., Frauenfelder, H. 1968. *Science* 160:438 (Abstr.)
57. Gunsalus, I. C., Grund, A., Yen, K.-M. 1982. In *Oxygenases and Oxygen Metabolism,* ed. M. Nozaki, et al, pp. 79–91. New York: Academic
58. Gunsalus, I. C., Gunsalus, C. F., Chakrabarty, A. M., Sikes, S., Crawford, I. P. 1968. *Genetics* 60:419–35
59. Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P. G., Münck, E. 1974. In *Molecular Mechanisms of Oxygen Activation,* ed. O. Hayaishi, pp. 559–613. New York: Academic
60. Gunsalus, I. C., Morton, R. B., Namtvedt, M. J., Sager, R. D. 1964. *148th Ann. Meet. Am. Chem. Soc., Chicago,* C51 (Abstr.)
61. Gunsalus, I. C., Sligar, S. G. 1978. *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:1–44
62. Gunsalus, I. C., Struglia, L., O'Kane, D. J. 1952. *J. Biol. Chem.* 194:859–69
63. Gunsalus, I. C., Wagner, G. C., Debrunner, P. G. 1980. In *Microsomes, Drug Oxidations, and Chemical Carcinogenesis,* ed. M. J. Coon, et al, pp. 233–42. New York: Academic
64. Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., Foust, C. E. 1945. *J. Biol. Chem.* 161:753–44
64a. Gunsalus, I. C., Wagner, G. C. 1978. In *Methods in Enzymology,* ed. S. Fleisher, L. Packer, 52:166–68. New York: Academic
65. Gunsalus, I. C., Yen, K.-M., 1981. In *Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids,* ed. S. B. Levy, R. C. Clowes, E. L. Koenig, pp. 449–509. New York: Plenum
66. Haniu, M., Armes, L. G., Tanaka, M., Yasunobu, K. T., Shastry, B. S., et al. 1982. *Biochem. Biophys. Res. Commun.* 105:889–94
67. Haniu, M., Tanaka, M., Yasunobu, K. T., Gunsalus, I. C. 1982. *J. Biol. Chem.* 257:12657–63
68. Haniu, M., Yasunobu, K. T., Gunsalus, I. C. 1983. *Biochem. Biophys. Res. Commun.* 116:30–38
69. Hanson, L. K., Eaton, W. A., Sligar, S. G., Gunsalus, I. C., Gouterman, M. et al. 1976. *J. Am. Chem. Soc.* 98:2672–74
70. Hedegaard, J., Conrad, H. E., Gunsalus, I. C. 1961. *Bacteriol. Proc.* 183:p66 (Abstr.)
71. Hedegaard, J., Gunsalus, I. C. 1965. *J. Biol. Chem.* 240:4038–43
72. Hornberger, C. S. Jr., Heitmiller, R. F., Gunsalus, I. C., Schnakenberg, G. H. F., Reed, L. J. 1952. *J. Am. Chem. Soc.* 74:2382
73. Inouye, S., Ebina, Y., Nakazawa, A., Nakazawa, T. 1984. *Proc. Natl. Acad. Sci. USA.* In press
74. Johnston, J. B., Gunsalus, I. C. 1977. *Biochem. Biophys. Res. Commun.* 75: 13–19
75. Katagiri, M., Ganguli, B. N., Gunsalus, I. C. 1968. *J. Biol. Chem.* 243:3543–46
76. Klein, S. M., Sager, R. D., Namtvedt, M. J., Gunsalus, I. C. 1964. *Bacteriol. Proc.* 91 (Abstr.)
77. Kline, L., Pine, L., Gunsalus, I. C., Barker, H. A. 1952. *J. Bacteriol.* 64:467–72
77a. Koga, H., Aramaki, H., Yamaguchi, E., Horiuchi, T. 1984. *J. Pharm. Dynamics,* in press
77b. Koga, H., Rauchfuss, B., Gunsalus, I. C. 1984. *J. Bacteriol.* In press
78. Korkes, S., Del Campillo, A., Gunsalus, I. C., Ochoa, S. 1951. *J. Biol. Chem.* 193:721–35
79. Lange, R., Hui Bon Hoa, G., Debey, P., Gunsalus, I. C. 1978. *Proc. Sci. Conf. on Cytochrome P450 Structural and Functional Aspects, Eberswalde, GDR, July 9–13* (Abstrs. 27–29) *Chem. Abstrs.* 91:86414b
80. Lange, R., Hui Bon Hoa, G., Debey, P., Gunsalus, I. C. 1979. *Acta Biol. Med. Ger.* 38:143–52
81. Lange, R., Hui Bon Hoa, G., Debey, P., Gunsalus, I. C. 1979. *Eur. J. Biochem.* 94:491–96
81a. Lipscomb, J. E., Gunsalus, I. C. 1973. In *Iron-Sulfur Proteins,* ed. W. Lovenberg, 1:151–72. New York: Academic
81b. Luria, S. E. 1984. *A Slot Machine, A Broken Test Tube.* New York: Harper & Row. 228 pp.
82. Lwoff, A., Lwoff, M. 1937. *Proc. R. Soc. London Ser. B* 122:352
83. Miller, A. K., Campbell, J. J. R., Gunsalus, I. C. 1947. *J. Bacteriol.* 54:22 (Abstr.)
83a. Monod, J. 1971. *Chance and Necessity.* New York: Alfred A. Knopf. 199 pp.
84. Murray, R. I., Fisher, M. T., Debrunner, P. G., Sligar, S. G. 1984. In *Metalloproteins,* ed. P. Harrison, Vol. 1: In press. London: McMallean
84a. O'Kane, D. E., Gunsalus, I. C. 1947. *J. Biol. Chem.* 170:425–32
85. O'Kane, D. J., Gunsalus, I. C. 1947. *J. Bacteriol.* 54:20 (Abstr.)
86. O'Kane, D. J., Gunsalus, I. C. 1948. *J. Bacteriol.* 56:499–506
87. Orla-Jensen, S. 1919. *The Lactic Acid Bacteria.* Copenhagen: I. Kommission Hos Ejnar Munksgaard
88. Orme-Johnson, W. H., Hansen, R. E., Beinert, H., Tsibris, J. C. M., Bartholo-

maus, R. C., Gunsalus, I. C. 1968. *Proc. Natl. Acad. Sci. USA* 60:368–72
88a. Ornston, L. N., Sligar, S. G., eds. 1982. *Experiences in Biochemical Perception*. New York: Academic
89. Ougham, H. J., Taylor, D. G., Trudgill, P. W. 1983. *J. Bacteriol.* 153:140–52
90. Pierre, J., Bazin, M., Debey, P., Santus, R. 1982. *Eur. J. Biochem.* 124:533–37
91. Poulos, T. L., Alden, R. A., Wagner, G. C., Gunsalus, I. C. 1984. *J. Mol. Biol.* In press
92. Poulos, T. L., Perez, M., Wagner, G. C. 1982. *J. Biol. Chem.* 257:10427–29
93. Queener, S. W., Queener, S. F., Meeks, J. R., Gunsalus, I. C. 1973. *J. Biol. Chem.* 248:151–61
93a. Rahn, O. 1932. *Physiology of Bacteria*. Philadelphia: P. Blakiston & Son. 438 pp.
94. Rahn, O. 1945. *Microbes of Merit*. Lancaster, PA: Cattrell. 277 pp.
95. Reed, L. J., Gunsalus, I. C., Schnakenberg, G. H. F., Soper, Q. F., Boaz, H. E., et al. 1953. *J. Am. Chem. Soc.* 75:1267–70
96. Sato, R., Omura, T., eds. 1978. *Cytochrome P450*. New York: Academic/Tokyo: Kodansha. 233 pp.
97. Sligar, S. G., Debrunner, P. G., Lipscomb, J. D., Namtvedt, M. J., Gunsalus, I. C. 1974. *Proc. Natl. Acad. Sci. USA* 71:3906–10
98. Sligar, S. G., Gunsalus, I. C. 1976. *Proc. Natl. Acad. Sci. USA* 73:1078–82
99. Sligar, S. G., Lipscomb, J. D., Debrunner, P. G., Gunsalus, I. C. 1974. *Biochem. Biophys. Res. Commun.* 61: 290–96
100. Snell, E. E. 1944. *J. Biol. Chem.* 154:313
101. Stanier, R. Y., Palleroni, M. J., Doudoroff, M. 1966. *J. Gen. Microbiol.* 43: 150–271
101a. Stryer, L., ed. 1981. *Biochemistry*, San Francisco: W. H. Freeman, pp. 293.
102. Tanaka, M. Haniu, M., Yasunobu, K. T., Dus, K., Gunsalus, I. C. 1974. *J. Biol. Chem.* 249:3689–701
102a. Taylor, D. G., Trudgill, P. W. 1984. *Proceedings of the 8th Int. Symp. on Flavins and Flavoproteins, Brighton, U. K., July 8–13*
103. Travers, F., Douzou, P., Pederson, T., Gunsalus, I. C. 1975. *Biochimie* 57:43–48
104. Trudgill, P. W., DuBus, R., Gunsalus, I. C. 1966. *J. Biol. Chem.* 241:1194–205
105. Trudgill, P. W., DuBus, R., Gunsalus, I. C. 1966. *J. Biol. Chem.* 241:4288–90
106. Tsibris, J. C. M., Namtvedt, M. J., Gunsalus, I. C. 1968. *Biochem. Biophys. Res. Commun.* 30:323–27
107. Tsibris, J. C. M., Tsai, R. L., Gunsalus, I. C., Orme-Johnson, W. H., Hansen, R. E., et al. 1968. *Proc. Natl. Acad. Sci. USA* 59:959–65
108. Ullah, A. H. J., Bhattacharyya, P. K., Bakthavachalam, J., Wagner, G. C., Gunsalus, I. C. 1983. *Fed. Proc.* 42: 1897
109. Vollmer, S. J., Switzer, R. L., Debrunner, P. G. 1983. *J. Biol. Chem.* 258: 14282–93
110. Wagner, G. C. 1981. *Fed. Proc.* 40:1663 (Abstr.)
111. Wagner, G. C., Gunsalus, I. C., Wang, M.-Y. R., Hoffman, B. M. 1981. *J. Biol. Chem.* 256:6266–73
112. Wagner, G. C., Perez, M., Toscano, W. A. Jr., Gunsalus, I. C. 1981. *J. Biol. Chem.* 256:6262–65
113. Wood, W. A., Gunsalus, I. C., Umbreit, W. W. 1947. *J. Bacteriol.* 54:21 (Abstr.)
114. Wood, W. A., Gunsalus, I. C., Umbreit, W. W. 1947. *J. Biol. Chem.* 170:313–21
115. Worsey, M. J., Franklin, F. C. H., Williams, P. A. 1978. *J. Bacteriol.* 134: 757–64
116. Yasunobu, K. T., Haniu, M., Gunsalus, I. C. 1984. In *Biochemical and Physical Studies of Proteins and Nucleic Acids*, pp. 305–15. New York: Elsevier
117. Yen, K.-M., Gunsalus, I. C. 1982. *Proc. Natl. Acad. Sci. USA* 79:874–78
118. Yen, K.-M., Sullivan, M., Gunsalus, I. C. 1983. *Plasmid* 9:105–11
119. Yu, C. A., Gunsalus, I. C., Katagiri, M., Suhara, K., Takemori, S. 1974. *J. Biol. Chem.* 249:94–101

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CYANOBACTERIAL CELL INCLUSIONS

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INTRODUCTION

Cyanobacteria (blue-green algae, blue-green bacteria, cyanophytes) are probably the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes (118). Unlike other photosynthetic bacteria, the cyanobacteria contain chlorophyll *a* and carry out plant-like oxygenic photosynthesis. They differ from the prochlorophytes (82) by not containing chlorophyll *b*. Their ecological importance has long been recognized in the process of eutrophication and in their ability to survive and emerge in varying as well as stressful habitats (26).

The study of other properties such as their prokaryotic nature, physiology, biochemistry, and genetics has only become a subject of increasing interest in

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the last 20 years. In particular, three books have brought together information on this group in the last ten years (26, 27, 44).

Cyanobacterial fine structure was first reviewed in Annual Review of Microbiology in 1968 (75); that review concluded that the state of knowledge about intracellular inclusions was: "the variety of inclusions identified in electron micrographs of blue-green algae is surpassed only by the number of names, chemical composition, and functional importance assigned to them." Six years ago Stanier & Cohen-Bazire (118) described the methodological revolution—the introduction of experimental analysis instead of descriptive analysis—since the first review and reviewed the organization of the cyanobacterial cell. Other than specialized chapters in the most recent book (27) on structure and function of particular subjects such as membranes (58), phycobilisomes (32), and cell outer surface (39), no review has emphasized the role of a variety of cellular inclusions since Shively's review of prokaryotic cell inclusions (107).

It is the intent of this review to summarize information on intracellular inclusions arising since 1974, and in particular, data from studies of environmental influences on ultrastructure. Because many of the cyanobacterial inclusions have reserve functions, they accumulate under starvation conditions as stores of nitrogen (cyanophycin, phycobilisomes), carbon and energy (polyglucose, poly-β-hydroxybutyrate), or phosphate (polyphosphate bodies). Other inclusions to be discussed are polyhedral bodies or carboxysomes, which contain ribulose-1,5-bisphosphate carboxylase (RUBPC'ase), and gas vacuoles. Ribosomes, which are dispersed throughout the cyanobacterial cytoplasm but are in highest density near the nucleoplasm, will not be discussed because they appear to be typical prokaryotic 70S ribosomes that dissociate into 50S and 30S subunits (128), and little has been determined on the process of ribosome formation and the regulation of their synthesis in cyanobacteria since last reviewed (38, 118). The intracytoplasmic membranes, containing the photosynthetic apparatus, will not be discussed because they, and the influence of the environment on their development, have been carefully reviewed (58) recently.

GENERAL CYANOBACTERIAL CELL ORGANIZATION

The general organization of the cyanobacterial cell has been reviewed (118), but the elegant recent work of Nierzwicki-Bauer et al (88–90) must be mentioned. These investigators have made the first three-dimensional ultrastructural reconstruction of an entire cyanobacterium with the aid of high-voltage electron microscopy (HVEM) and computer-aided assembly of serial sections.

Thick sections of the unicellular *Agmenellum quadruplicatum* (0.25–10 μm) could be viewed with little loss in resolution; the three-dimensional arrangement of subcellular features was very consistent from one cell to another. In

particular, nuclear material, polyphosphate bodies (average 5 per cell), and carboxysomes (average 6 per cell) were located only in the center of the cell, never among the peripheral system of photosynthetic membranes that compartmentalized the central cytoplasmic region. Occasionally contact was seen between polyphosphate bodies and carboxysomes. Usually ribosomes were also confined to the central cytoplasm in areas not filled by inclusions. Glycogen granules were dispersed between thylakoids throughout the periphery; lipid bodies (17 average per cell) were always positioned between the outermost pair of thylakoid membranes and the cytoplasmic membrane at the periphery of the cell. No cyanophycin was observed, probably because cells were in logarithmic growth where cyanophycin level is low; cyanophycin has been seen in *A. quadruplicatum* only when starved for phosphorus (121). The defined location of inclusions under exponentially growing conditions allows some functional speculations that must be tested in other organisms and in cells grown under different growth conditions where cell ultrastructure varies (120, 121).

That carboxysomes are never seen in contact with thylakoids might suggest storage function instead of direct involvement in CO_2 fixation. Contact between carboxysomes and thylakoids has been seen, however, in *Anabaenopsis sp.* (97). Since carboxysomes and polyhedral bodies only occasionally are observed to contact each other, there is probably no functional significance to the contact (90). Storage function of polyglucose granules is consistent with their location only between thylakoid membranes.

The photosynthetic membrane of *A. quadruplicatum* consists of four to six pairs of thylakoid sheets traversing the entire longitudinal axis of the cell, completely surrounding the central cytoplasmic region with an interconnecting network of concentric shells. The thylakoids merge only with the inner surface of the cytoplasmic membrane. Whether these contacts are sites of thylakoid synthesis, are ways of maintaining the three-dimensional arrangement of the thylakoid system, or whether they facilitate segregation of nuclear material or facilitate transport (90) must still be determined.

EFFECTS OF ENVIRONMENTAL CHANGE ON CELLULAR INCLUSIONS

Certain inclusions are always present in cyanobacterial vegetative cells: phycobilisomes, carboxysomes, glycogen granules, and polyphosphate granules. Cyanophycin granules are found in most species, and gas vacuoles and poly-β-hydroxybutyrate granules are present in a minority of species and under certain conditions. Rapid accommodation of populations of cells caused by shifts in environment, both in nature or in the lab, is effected by changes in structure and function in individual cells (129); it is therefore necessary to

specify environmental conditions when defining a microbe's structure and function.

In order to extrapolate results of experiments in the laboratory to what occurs in natural environments, the influence of variable environmental factors or nutrient-sufficient and nutrient-limited cultures must be determined. The presence of reserve materials such as the nitrogen reserves, phycocyanin and cyanophycin, give cyanobacteria a potential selective advantage. This would be especially true for species that do not fix nitrogen or for those adapting to nitrogen fixation, since combined nitrogen is likely to be a limiting nutrient for growth of cyanobacteria in natural environments (134). Natural populations of *Microcystis flos-aquae* show large variations in the amount of cyanophycin (122); accumulation is seen in cells before and after a bloom.

Experiments using nitrogen- or phosphorus-limited continuous cultures have given no meaningful relationship between growth rate and external nutrient concentration (64); this could in part be due to the role of endogenous reserve materials in supplying nitrogen and phosphorus. The relevance of laboratory culture studies to growth in natural ecosystems has been recently reviewed (129), as has the control of genomic expression related to many effects of environmental variation (38). Each inclusion will be discussed below in relation to its occurrence and amount as a cell adapts to new environments.

Carboxysomes

Polyhedral bodies (95) or carboxysomes (108) contain the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBPC'ase), a key determinant of the capacity of organisms for the net assimilation of inorganic carbon. Carboxysomes are large structures (0.1–0.7 μm) that exhibit RUBPC'ase activity and react with RUBPC'ase antiserum (30). Detailed examinations have been carried out with only a few species, such as the the filamentous *Anabaena cylindrica* (30) and *Chlorogloea fritshii* (74) and the unicellular *Coccochloris peniocystis* (33), although they have been found in all strains of cyanobacteria observed (123). All 15 nitrogen-fixing strains observed (123) contained polyhedral bodies in vegetative cells and akinetes, but not in heterocysts, which do not fix CO_2 photosynthetically.

Carboxysomes were confined to cells in a certain region of the trichome in the nonheterocystous aerobic nitrogen-fixing *Oscillatoria erythraeae;* they were absent from cells in a region displaying high reducing potential (18). This agrees with the concept of physiological segregation of photosynthesis and nitrogen fixation. No visually apparent differences were seen in pigmentation of the granulated and nongranulated regions (18), but other data suggest lack of CO_2 fixation in the nongranulated regions (23). This strongly supports an hypothesis of a modified photochemical apparatus in the noncarboxysome-containing cells.

Two fractions—one soluble and one particulate—of RUBPC'ase activity have been found when cell extracts are separated by sucrose gradient centrifugation (30, 33). Twenty percent of the enzyme activity and 41% of immunoprecipitable activity of RUBPC'ase were measured in vitro in particulate fractions of late logarithmic cultures of *Anabaena cylindrica* in which polyhedral bodies were not abundant (30). Because these cells were broken mechanically, this may have led to breakage of the fragile carboxysomes, release of RUBPC'ase, and a subsequent increase in soluble enzyme activity relative to the particulate form.

A survey of ten species using isolation techniques and stabilization procedures that maintain the carboxysome structural integrity suggested 84–93% of the RUBPC'ase activity was in the particulate fraction (33). Mechanical lysis of sphaeroplasts or of whole cells resulted in a significant decrease in the amount of particulate enzyme and an increase in the soluble enzyme, suggesting that mechanical cell disruption may result in lysis of carboxysomes. Both soluble and particulate enzymes are of similar molecular weight, and in most cyanobacteria the enzyme has been shown to consist of both large (M_r 53,000) and small (M_r 13,000) subunits (74).

Tabita's studies (i.e. 125) consistently show the presence of purified active enzymes containing only eight large subunits, while most other studies (except 29, in which the enzyme was obtained from an anoxygenic photosynthesizer that contained only four large subunits) suggest an enzyme with eight large and eight small subunits. Preparation and purification conditions are very important because RUBPC'ase from *Aphanocapsa* 6308 consisted of both subunit sizes when acid precipitation was not carried out; acid precipitation purification produced an enzyme containing only large subunits (31). Repeated acid precipitation of a marine *Synechococcus* purified enzyme resulted in loss of the small subunit and loss of activity (8). Electron microscopically observed ring-shaped structures about 12 nm in diameter have the external dimensions of RUBPC'ase molecules (74).

Carboxysome function is still unknown, with possibilities having been suggested of activity in CO_2 fixation or of enzyme storage reserve (30, 107). The fact that carboxysomes occur in akinetes that do not fix CO_2 photosynthetically supports their possible storage function (123), although finding that RUBPC'ase activity per mg RUBPC'ase protein of the carboxysomal enzyme was higher or equal to the enzyme present in the cytosol, and that carboxysomes do not serve as nitrogen storage compounds, make it unlikely that they serve as storage bodies (12). However, the symbiotic cyanobacteria of liverworts contain polyhedral bodies, but do not fix CO_2 (103), suggesting a storage function for these inclusions.

Purified carboxysomes of the facultative chemolithotroph *Thiobacillus neapolitanus* contain all the relevant Calvin cycle enzymes as well as malate

dehydrogenase, aspartate aminotransferase, and adenylate kinase (13). Previous unsuccessful attempts to detect other Calvin cycle enzymes in purified carboxysomes may be due to their latency, requiring unmasking of activity by treatment such as sonication. A model for carboxysome functioning in autotrophic prokaryotes has been proposed for both CO_2 fixation and for a shuttle mechanism for NADH transport to ensure high concentrations of NADH required for CO_2 fixation within the carboxysome (13).

A new purification procedure resulting in stable, homogeneous carboxysomes from *T. neapolitanus* was recently described (22); ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, and fructose-1,6-bisphosphatase could not be detected in these carboxysomes, but 12–15 polypeptides (two being associated with the shell) were present. These data suggest rejection of the proposed "Calvinsome" model of Beudeker & Kuenen (13). Use of similar techniques to prepare purified, stable carboxysomes should make it possible to undertake better characterization of cyanobacterial carboxysomes, both in terms of function and in terms of structure, and to determine whether the small subunit is a normal component of the enzyme lost in preparation or whether it is acquired during isolation.

CO_2 starvation did not cause a decrease in carboxysomes in *Synechococcus lividus* (87), but high white light-grown cells had significantly fewer carboxysomes (but relatively high RUBPC'ase activity) than cells grown in red light or in white light of low intensity. In these experiments starvation for CO_2 was imposed by bubbling the supplied air through NaOH traps; recently (106) different conditions were used in comparing iron-deficient *Anacystis nidulans* that were considered starved (gently shaken) or not starved for CO_2 (0.03% CO_2 = air bubbled through cultures). Low-iron cells showed a decrease in carboxysomes and rare contact with thylakoids. The number of carboxysomes increased rapidly when iron was added to gently shaken cells, and the carboxysomes became associated with the nucleoplasmic face of the inner photosynthetic membrane; carboxysomes remained low and rarely contacted thylakoids when iron was restored to cells bubbled with air.

That carboxysomes were more prevalent in shaken cells and that they contact the thylakoids suggests carboxysome function in CO_2 fixation. Low-iron cells contain large amounts of glucose storage granules that could be utilized in place of CO_2 fixation; increase in carboxysomes was seen when glucose storage granules disappeared. No observations were reported for noniron-starved bubbled cells, so no comparisons can be made on the effect of CO_2 levels alone on cell ultrastructure. It would be of interest to compare the 0.03% CO_2-bubbled cells to both shaken and higher CO_2 concentration–bubbled cells and to levels of CO_2 fixation in all types of cells, in order to reveal if carboxysomes are required for optimal CO_2 fixation. It is unlikely that 0.03% CO_2 is CO_2-sufficient, so the number of carboxysomes may decrease even more in cells grown with higher CO_2 concentrations.

Carboxysomes persisted during phosphorus starvation (121), but reports of changes during nitrogen starvation are conflicting. Carboxysomes were degraded in *Anabaena variabilis* (35) and *Spirulina platensis* (132) but unaffected by nitrogen limitation in *Agmenellum quadruplicatum* (120). More studies on the effect of CO_2 and nitrogen starvation on polyhedral bodies are necessary to clarify their fate and to ascertain whether effects are species-dependent.

The air pollutant SO_2, which has effects on lichens, affected the *Nostoc* partner of the *Peltigera canina–Nostoc* symbiosis by decreasing the number of carboxysomes up to 74% as the concentration of SO_2 increased from 9 ppm to 100 ppm (105); the decrease was not due to akinete initiation, but likely was a direct effect of SO_2 on RUBPC'ase synthesis. The inhibiting effect of SO_2 on the photosynthetic rates in lichens has been widely reported (62).

Although RUBPC'ase levels were shown to depend on light intensity in *Anacystis*, only two light intensities were tested (36). Other investigators, using magnesium limited chemostats (Utkilen & Briseid, unpublished data), suggest RUBPC'ase content is independent of light intensity down to 80 $\mu E\, m^{-2}s^{-1}$; a further decrease in light intensity resulted in a decrease in the enzyme.

Phycobilisomes

Phycobiliproteins (PBP) are the most abundant proteins in the cyanobacterial cell and, together with a number of colorless linker polypeptides (127), are typically found in phycobilisomes, which are disc-shaped bodies with a cross-sectional diameter of 20–70 nm attached in regular array along the thylakoid surface. Only in *Gleobacter* is this arrangement not observed; this cyanobacterium, unique in lacking thylakoids, contains phycobiliproteins as a continuous, electron-dense, cortical layer (80 nm thick) of bundle-shaped phycobilisomes right beneath the cell membrane (60, 102). In *Synechococcus* 6301 over 95% of the phycobiliproteins are located in phycobilisomes (148).

Phycobilisomes are composed of the antennae or light-harvesting phycobiliprotein pigments that absorb energy for high-efficiency transfer to photosystem II. Phycobiliproteins utilize linear tetrapyrroles (bilins) as chromophores and are all composed of two dissimilar subunits, α and β, with molecular weights of 14,000–20,000. Five major classes of phycobiliproteins have been described: allophycocyanin B, allophycocyanin, phycocyanin, phycoerythrocyanin, and phycoerythrin (17). All cyanobacteria contain phycocyanin, allophycocyanin, and probably allophycocyanin B; the other two pigments are present in some cyanobacteria (17).

The attachment of phycobilisomes to the outer surface of thylakoids is believed important for high efficiency transfer of energy stepwise from phycoerythrin or phycocyanin to allophycocyanin and chlorophyll *a* at the reaction center of photosystem II. Details of phycobilisome structure and function have been frequently reviewed recently (32, 46–48, 55, 56).

Many experiments have added information to a model (17) for the hemidiscoidal phycobilisomes, which suggests the structure consists of a central core of two or three discs of allophycocyanin seen in face view, with radiating arms of six hexameric rods of phycocyanin and phycoerythin seen in edge view. The model accounts for many aspects of phycobiliprotein assembly and energy transfer (17).

Plane of section markedly affects the appearance of phycobilisomes in thin sections. They are seen in face view when rows are cut in cross section in a plane perpendicular to the thylakoid surface, and appear semicircular in outline with the flat side attached to the thylakoid membrane. Edge view phycobilisomes on the thylakoid membrane are observed when the rows of phycobilisomes are cut in longitudinal section in a plane normal to the thylakoid surface or when rows are cut in tangential section. In both these latter cases, stacks of discs 6–10 nm in thickness are seen.

The unusual cyanobacterium *Gleobacter violaceus,* which lacks typical internal thylakoids, contains phycobilisomes with different structure. Each phycobilisome consists of six rods (50–70 nm long) attached probably to a flat plate of allophycocyanin, not a triangular core (60).

Temperature (20–30°C) and high ionic strength (0.75 M, pH 6.8) have been shown to be the most crucial factors in maintaining phycobilisome structure in vitro (49). The close packing of phycobilisomes on the thylakoid surface may substitute for the high phosphate required in solution (110).

Recent experiments accomplishing in vitro reconstitution of phycobilisomes, both intragenerically (21) and intergenerically (20), open up the possibility of preparing phycobilisomes lacking one or more specific polypeptides in order to explore their role in energy transfer and in phycobilisome structure and assembly. Phycobilisomes are a heterogeneous population of structures, because the phycobilisome content of cyanobacteria and the relative amounts of their various light-harvesting components depend on light quality and intensity, age of culture, and its nutritional state.

In many freeze-fractured preparations of cyanobacteria, intramembrane particles of 10 nm diameter on the exoplasmic fracture (EF) face are arranged in a pattern similar to that of phycobilisomes on the membrane surface (34, 58, 83). This has suggested that EF particles contain reaction centers for photosystem II (51) within the membrane and that they are in direct contact with phycobilisomes on the thylakoid surface, because the majority of light energy absorbed by phycobiliproteins is transferred initially to photosystem II (142). EF particles are absent from thylakoids in cyanobacterial heterocysts that lack photosystem II (51). The EF particles therefore probably represent photosystem II complexes, suggesting that phycobilisomes are positioned on the thylakoid membrane in direct contact with photosystem II centers within the thylakoid membrane (52).

Oscillatoria splendida has one phycobilisome per EF particle, while *O. brevis* and *O. limosa* have two (83). All three species have the same size and number of phycobilisomes but in the latter two the EF particles are much larger and half as numerous as in *O. splendida*. Therefore, larger EF particles have been suggested to contain two photosystem II reaction centers (34), and each of the three species would have about the same size antennae serving each reaction center. In *Gleobacter* (34), antenna size per reaction center must be much smaller.

LIGHT QUALITY EFFECTS Many but not all cyanobacteria containing phycoerythrin undergo complementary chromatic adaptation (reviewed in 14, 126a) in which rates of phycoerythrin (PE) and phycocyanin synthesis are affected by the wavelength of light to which cells are exposed during growth. The chromatically induced modification of the phycoerythrin:phycocyanin ratio involves new protein synthesis, not turnover (11), and appears to involve gene regulation at the transcriptional level (50).

Two types of adaptive responses have been described (126). In certain cyanobacteria only the rate of phycoerythrin synthesis is regulated; the rate of phycoerythrin synthesis is maximal in green light and minimal or none in red light. In other cyanobacteria both phycoerythrin and phycocyanin syntheses are regulated; in green light the rate of phycoerythrin synthesis is maximum and that of phycocyanin synthesis minimum. In red light phycocyanin is maximally synthesized, while phycoerythrin is minimally synthesized or synthesis stops.

This increased rate of phycocyanin synthesis reflects expression of phycocyanin structural genes that are not expressed during growth in green light (16). Phycobilisomes isolated from *Synechocystis* 6701, which regulates the rate of phycoerythrin synthesis alone, and of LPP-7409, which regulates the rate of synthesis of both phycoerythrin and phycocyanin, have been analyzed by electron microscopy (17). Widely different phycocyanin:phycoerythrin ratios have no effect on the gross structure of phycobilisomes. Phycobilisomes from *Synechocystis* 6701 grown in red light were smaller than those from cells grown in green light. LPP-7409 phycobilisomes from cells treated both ways were similar in size.

In no case did the triangular core vary in size or thickness; however, the change in length of the peripheral rods was responsible for the size variation, changing from 2.20 discs in phycobilisomes from red light *Synechocystis* 6701 to 3.60 discs per rod in LPP-7409 (17). Phycobilisomes from photoregulated *Fremyella diplosiphon,* which differ markedly in the amounts of both phycoerythrin and phycocyanin relative to allophycocyanin, were first examined by Rosinski et al (104). The structure of phycobilisomes from cells grown in red light and from cells grown in white light were similar; the largest phycobili-

somes from red-light–grown cells had a maximum of three discs per arm while those from white-light–grown cells had a maximum of four discs per arm. In all phycobilisome samples examined, there was some variation in the number of discs in the peripheral arms (110). The function of each linker polypeptide in phycobilisome assembly has recently been identified in *Nostoc* (sp) (151).

LIGHT QUANTITY EFFECTS Ohki & Gantt (91) recently studied the heterotroph *Tolypothrix tenuis*, which synthesizes PE in the dark; dark-synthesized phycobiliproteins are organized in energetically functional phycobilisomes, and their morphology is the same as red-light phycobilisomes. Isolated phycobilisomes had a central core of three units and six peripheral rods, each composed of three hexamers. Thin sections showed that phycobilisomes were attached to thylakoid surfaces at a ratio of 60 phycobilisomes per 1000 nm of thylakoid length.

In *Anabaena variabilis* the efficiency of energy transfer from phycobiliproteins to photosystem II was low in dark-grown cells even though both phycobiliproteins and photosystem II were synthesized in the dark (92). Prolonged growth of *Chlorogloea fritschii* in the dark resulted in a 20-fold reduction in phycobiliprotein content (41). A functional light-harvesting system synthesized even in the dark can be regarded as advantageous to a cyanobacterium's survival, since there will be no lag in photosynthetic activity when cells are transferred to the light.

A unialgal culture growing normally at one lux of light on a cave wall was recently obtained and observed ultrastructurally (34). Phycobilisomes were difficult to see, but appeared unusually large; absorption spectra showed phycobilins were present but extraction was not possible (34). Large antennae could be associated with a low-light habitat. Fewer phycobilisomes and less phycocyanin have been observed in cells grown in high white light than in red or low white light (36, 37, 131).

Phycobiliprotein composition of dark-grown facultatively heterotrophic strains that adapt chromatically was the same as phycobiliprotein composition of cells grown in red light (126). The total rate of phycobiliprotein synthesis remained high during dark growth.

Heterocysts, which do not have an active photosystem II and do not evolve O_2, have been reported to lack phycobilisomes (1); the presence of about one half to one third the phycocyanin content of vegetative cells, and insignificant amounts of allophycocyanin have been found in purified heterocysts from *A. variabilis* (99). Other heterocysts have recently been shown to contain one half the phycocyanin level of vegetative cells and detectable levels of allophycocyanin (73). Purified phycocyanin and allophycocyanin from whole filaments and heterocysts appeared identical (73).

The quantum efficiency of the phycocyanin in *A. variabilis* heterocysts is 50–70% of that of chlorophyll *a* in sensitizing P700 oxidation of photosystem I

(99). Energy transfer from phycobiliproteins to photosystem I may be a function of phycobiliproteins in heterocysts. The photosynthetic unit (chlorophyll *a*/P700) is modified during differentiation of heterocysts in order to support the specialized energetic requirements of the nitrogen-fixing cells (1).

NUTRIENT STARVATION AND REPLETION Allen & Smith (6) were the first to suggest, from quantitative experiments, the possibility that phycocyanin might be a nitrogen storage compound in cyanobacteria; cellular levels of phycocyanin decreased markedly when cells were deprived of a source of combined nitrogen. This loss of phycocyanin has been noted often (15, 35, 77, 94, 96, 120, 134, 142, 148). As cells depleted of phycobiliproteins can continue to photosynthesize because sufficient chlorophyll *a* remains associated with photosystem II (81), phycobiliproteins are not indispensible. They therefore represent a potential pool of amino acids to be utilized with no irretrievable damage to a nitrogen-starved cell.

The most striking change in appearance of nitrogen-starved cyanobacteria is their change in color from blue-green to yellow-green due to phycobiliprotein degradation. Lau et al (77) presented evidence that spectrophotometric decrease of phycocyanin upon nitrogen starvation reflected coordinate degradation of the two phycocyanin apoprotein subunits and repression of new phycocyanin synthesis. This decrease was shown by disappearance of phycobiliprotein bands from acrylamide gels after electrophoresis (42, 77, 147) and by the loss of immunologically reactive material (77).

Protein pulse–labeling and methionine sulfoxamine (MSO) treatment also suggested cyanobacteria rapidly degrade proteins (phycocyanin being the major form of protein hydrolyzed) in response to nitrogen starvation and MSO treatment and utilize the amino acids released for new protein synthesis (94). As long as phycocyanin content did not decrease below 50%, growth continued at normal rates (15). Phycobilisome turnover during nitrogen starvation in *Synechococcus* 6301 was indicated by a decrease in their sedimentation coefficient, a decrease in the phycocyanin:allophycocyanin ratio, a shift in fluorescence emission maximum from 673 to 676 nm, and a selective loss of a 30 kd nonpigmented polypeptide after 24 hours of starvation (148). After 72 hours of nitrogen starvation the intracellular level of phycocyanin decreased more than 30-fold.

The results indicated that free phycobiliproteins were degraded; also, a significant portion of the phycocyanin from the periphery of the phycobilisomes was degraded during early stages of nitrogen starvation. Phycobilisomes partially depleted of phycocyanin still functioned efficiently in energy transfer (148). Since phycocyanin is in a position peripheral to allophycocyanin and allophycocyanin B in models for the location of individual phycobiliproteins in phycobilisomes and is the first pigment in the sequence for transfer of absorbed

light energy, partial loss of phycocyanin need not lead to a decrease in the effectiveness of the residual structure in energy transfer. Extended nitrogen starvation resulted in total degradation of residual phycobilisomes, possibly in conjunction with their detachment from thylakoids (148). Both nonspecific and specific phycobiliprotein proteases have been reported (15, 45, 146, 147), but in no instance has the enzyme been isolated and characterized.

Some data suggest significant kinetic differences in the rates of proteolytic degradation of different phycobiliproteins within the cell, and at least in *Synechococcus* 6301 protease(s) show no strict specificity for phycobiliproteins, because nonpigmented linker polypeptides are also degraded during phycobilisome breakdown (148). When protein synthesis is halted with chloramphenicol or by starvation of a methionine auxotroph for methionine, breakdown of phycobilisomes does not occur (148). Regulation of cellular phycocyanin level can occur two ways—by variation in either size or number of phycobilisomes; both have been seen during nitrogen starvation (148).

A recent ultrastructural analysis (120) showed that phycobilisomes were the first components of *Agmenellum quadruplicatum* to deteriorate during nitrogen limitation, supporting the importance of phycobiliproteins as a nitrogen reserves. The phycobilisome, therefore, acts not only as a major absorber of light in photosynthesis, but also plays a second role as a rapidly utilizable store of nitrogen.

Addition of a nitrogen source allows reversal of starvation conditions (3, 6, 35, 120); phycocyanin recovery began 3–5 hrs after addition of nitrogen to starved cultures (3, 6, 120), net protein synthesis began [and glycogen was utilized (6, 8, 120)] almost immediately after addition (3, 6). The rapid turnover suggests glycogen served as an endogenous carbon source for resynthesis of protein.

Phosphorus deficiency has also been reported to decrease the cellular phycocyanin content (121, 134). Limitation of CO_2 also has an effect on the phycocyanin content of cyanobacteria. When *A. nidulans* was grown on 0.03% CO_2, less phycocyanin was formed than when cells were grown on 1.0% CO_2 (40), suggesting control of quanta input to maintain photoreactions at a rate comparable with the dark reactions. Experiments comparing phycocyanin content to phycobilisome content in *Synechococcus lividus* (87) suggested cells starved 102 hours were devoid of phycobilisomes but had detectable levels of phycocyanin until 120 hours of starvation, when both chlorophyll *a* and phycocyanin were undetectable. When CO_2-enriched air was reintroduced, there was a rapid initiation in synthesis of both chlorophyll *a* and phycocyanin. Growth did not resume until pigment levels reached normal levels.

These experiments suggest phycocyanin might serve as a reserve carbon source under CO_2 starvation in a manner comparable to its acting as a nitrogen reserve under nitrogen starvation. Changes in CO_2 concentration, light intensity, and temperature caused widely varying ratios of phycocyanin to chlorophyll in *Anacystis nidulans* (57).

The effects of iron deficiency and iron restoration have recently been studied (106). The phycocyanin content and quantities of phycobilisomes were depressed in iron-deficient cells; addition of iron led to their rapid restoration. Phycobilisome assembly was seen by six hours after iron addition, and the number of phycobilisomes increased throughout restoration.

PIGMENT MUTANTS Pigment mutants have been observed and sometimes isolated, but rarely investigated. This is likely due to a high reversion frequency (119). Preliminary ultrastructural observations on a yellow-green mutant of *Anacystis nidulans,* with normal chlorophyll concentration but half the phycocyanin content of the wild type, suggested fewer phycobilisomes (119). That this mutant had the same growth rate and photosynthetic efficiency as the wild-type strain suggests other functions for phycocyanin.

Phycobiliprotein mutants of *Synechococcus* 6701 (a chromatically adapting strain) with low reversion rates (reversion frequency less than 10^{-9}) have been recently analyzed for phycobilisome structural differences (54, 145) in both white and red light. The structure of the core is unaltered, but the amount of phycocyanin or phycoerythrin relative to allophycocyanin appears to determine the number of rods per core, the number of discs per rod, and the amount of certain colorless proteins involved in phycobilisome assembly.

Phycobilisome mutants of *Synechococcus* 6301, a nonchromatically adapting strain not containing phycoerythrin, make incomplete but still functional phycobilisomes (150). Phycobilisomes were devoid of the 33-kd and 30-kd linker polypeptides and much of their phycocyanin. It was concluded that the 33-kd and 30-kd polypeptides link phycocyanin into discs, that the 27-kd polypeptide assembles with phycocyanin to form discs that terminate rod growth, and that the 75-kd polypeptide is associated with the phycobilisome core (85). The regulation of phycobilisome size is at the level of phycocyanin assembly, as these mutants showed (149). The extent of assembly depends on the presence and amount of both 30-kd and 33-kd polypeptides.

Cyanophycin Granules

Although often believed to be universally present in the vegetative cells of cyanobacteria (111, 118), cyanophycin has been shown to be absent from some species (29; Allen, unpublished); data support the hypothesis that it is unique to the cyanobacteria. Even in the presence of chloramphenicol, which induces granule formation (4, 113), none has been observed in the colorless sulfur bacteria that are morphologically similar to cyanobacteria (79). Cyanophycin (multi-L-arginyl-poly- [L-aspartic acid], arg-poly[asp] or cyanophycin granule polypeptide, CGP) accumulates in granules as cells approach stationary phase

of growth (4, 12), often in a size large enough to be resolved by light microscopy (7).

First isolated and characterized by Simon (111), cyanophycin was shown to correspond morphologically to the "structured granules," long familar to electron microscopists, that are variable in shape and size with a radiating pattern of substructure (76). Cyanophycin has been shown to be a high-molecular-weight nonribosomally synthesized polymer (113), consisting of equimolar quantities of arginine and aspartic acid arranged as a backbone of polyaspartate, with an arginine moiety attached to the β-carboxyl group of each aspartate by its α-amino group (116).

Cyanophycin is insensitive to commercially available proteases (116). That it is not broken down by clostridiopeptidase B (115), which specifically attacks the carboxyl peptide linkage of arginine, supports the conclusion that arginine is amino-bonded in the polypeptide. The protein is insoluble at neutral pH and low ionic strength, but is soluble in dilute acid or base (112). When dissolved in 6.0 M urea and isoelectrofocused, a major band is produced at pH 4.75 and a minor band at pH 6.10 (116). Circular dichroism and laser Raman spectroscopy studies suggest the insoluble form of the granule is similar, but not identical to the acid soluble form; the ordered structure is environmentally sensitive (115).

ACCUMULATION AND SYNTHESIS Cyanophycin is at low levels in rapidly growing cyanobacteria but accumulates when cells are starved for light (4, 132), phosphorus (4, 79, 121), or sulfur (4, 79) or when cells are grown at low temperature (131). Induction is caused by specific inhibitors of macromolecular synthesis such as rifampicin and 5-methyltryptophan (79), as well as by excess nitrogen-containing compounds (4, 79). Variable induction with different species and different amino acids has been reported (4, 79), possibly due to limited uptake and incorporation (7) or to differential permeability of the amino acids (79).

Data also suggest that many strains show spatial localization of cyanophycin, which changes little even under conditions where massive deposition occurs (79). That cyanophycin was not caused simply by a slow division rate is suggested by the fact that cells grown in continuous culture with light as the sole limiting nutrient, at a specific growth rate of 0.04 hr^{-1}, formed no visible granules, and the cyanophycin content was at background level (4).

Simon (114) isolated and purified 92-fold from the filamentous cyanobacterium *Anabaena cylindrica* an enzyme which incorporates arginine and aspartic acid into arg-poly(asp) in a reaction that requires ATP, KCl, $MgCl_2$, a sulfhydryl compound, and a primer for activity. The reaction is insensitive to the addition of protein synthesis inhibitors and to the addition of nucleases. This supported conclusions from in vivo studies (113) that the polypeptide is

synthesized nonribosomally. The reaction product has the same bands after isoelectrofocusing and the same size distribution (25–125 kd) on SDS acrylamide gels as that synthesized in vivo (111), although three weight classes of 39 kd, 67 kd, and 100 kd were observed in vitro (114). Incorporation of L-arginine is almost completely dependent on the presence of L-aspartate.

Stevens et al (121) hypothesized that cyanophycin is synthesized from internal conversion of cellular protein; this hypothesis is based on the decrease in amounts of total cellular nitrogen and phycocyanin at the same time as the number per cell section and diameter of granules increased with phosphorus starvation. Control mechanisms regulating levels of phycobiliproteins and cyanophycin appear different because in phorphorus-limited cultures phycocyanin is degraded but cyanophycin accumulates (4, 120).

Protein breakdown was also suggested as the source of arginine and aspartate used in cyanophycin synthesis in *Aphanocapsa* (2) from analysis of ^{14}C- and ^{3}H-labeled cells transferred to unlabeled medium and from analysis of pulse-labeled cells. Cyanophycin accumulation when chloramphenicol is added to cells is accompanied by decrease in cell protein (2–4). Furthermore, only a small amount of the isotope in $^{14}CO_2$ is incorporated into cyanophycin when cells are producing large quantities of cyanophycin (4).

NUTRIENT STARVATION AND REPLETION That cyanophycin serves as a cellular nitrogen reserve was suggested by Simon's data (112) on cyanophycin accumulation in stationary phase cultures of *Anabaena* and its rapid depletion upon growth after dilution. Cyanophycin content, like phycocyanin content, is at low level in nitrogen-limited cells (3, 4).

Kinetic shift-up (to higher light intensity in nitrogen-replete medium) and shift-down (to minus nitrogen or anaerobic conditions) experiments were carried out in *Aphanocapsa* 6308 to determine kinetic changes in nitrogen storage proteins (4). *Aphanocapsa* 6308 that was shifted up to high light or down to nitrogen-free medium showed decrease in ten hours in the amount of cyanophycin from between 2 and 3% to between 0.6 and 0.7%, a decrease more rapid than the increase in cellular dry weight. During the first two hours after shift the cyanophycin content decreased linearly by 29%, whereas the protein and phycocyanin concentrations did not fall. Phycocyanin concentration did not decrease until four hours after nitrogen was depleted from the medium; overall protein content did not decrease until six hours after nitrogen depletion (3).

Cyanophycin therefore appears to be degraded before phycocyanin. Phycocyanin also functions as a photosynthetic accessory pigment. Analyses of concentrated culture medium after shift-down experiments of *Aphanocapsa* to minus-nitrogen conditions suggest no arginine or arginine-containing peptides are excreted (4).

Cyanophycin concentration in *Aphanocapsa* 6308 increases immediately upon addition of a utilizable nitrogen source, rises to 5–12% of the dry weight of the cell 8–12 hours after addition, and then rapidly decreases (3). If chloramphenicol is added at the same time as nitrogen is added to a nitrogen-starved culture, cyanophycin reaches the highest levels observed (3). The rapid decrease in cyanophycin level soon after addition of nitrogen to a nitrogen-starved culture suggests a degrading system that is not synthesized during chloramphenicol treatment.

An exopeptidase, cyanophycinase, has been reported in the nitrogen-fixing filamentous *Anabaena* (61). It has a pH optimum of 8.5, has no cation requirements, was inhibited by L-arginine and L-aspartate, and produced an asp-arg dipeptide as product. A higher activity of the enzyme was found in heterocyst extracts than in vegetative cell extracts. Another peptidase has been identified recently in *Aphanocapsa* (5); this enzyme effects degradation of ^{14}C-labeled cyanophycin into aspartate and arginine. Activity was seen in extracts of cells harvested 16–24 hours after nitrogen recovery began in nitrogen-limited cells.

The catabolic products of arginine metabolism were observed in *Aphanocapsa* 6308 by thin layer chromatography, by limiting growth conditions, and by enzymatic analysis (143). The presence of an arginine dihydrolase pathway allows the formation of ATP, ammonia, CO_2, and ornithine; the CO_2 produced from arginine appears to be photosynthesized for growth. An arginase pathway is also present in *Aphanocapsa* and utilized for nitrogen conservation, but no arginine decarboxylase pathway was observed. Arginine can therefore serve as a nitrogen source (ammonia) as well as a carbon source (CO_2) for *Aphanocapsa*. The enzyme systems do not appear to be inducible (143). The presence of this pathway makes net ATP systhesis from cyanophycin a possibility not so far tested.

Cyanobacteria starved for CO_2 had low cyanophycin content (4, 87) in comparison to high content when cells were given 5% CO_2. Because enzymes of the arginine dihydrolase pathway were present in *Aphanocapsa* (143), arginine from cyanophycin could be converted to CO_2, which might be incorporated into products of photosynthesis or other carboxylation reactions in the cell. Under CO_2-limiting conditions, cyanophycin may serve as a reserve carbon source.

Accumulation of cyanophycin granules (and polyglucose granules) are normal features during akinete differentiation (124). SO_2 treatment has an effect on cyanophycin formation in a *Nostoc* partner in a lichen (105). In 10 ppm SO_2, granule size but not number increased, whereas in 100 and 1000 ppm, both size and number increased significantly, and some cells differentiated into akinetes, suggesting $S0_2$ acts as a stress factor leading to cyanophycin and akinete formation.

Polyphosphate Granules

Polyphosphate granules (metachromatic granules or volutin) are 0.1–0.3 μm spherical aggregates of high-molecular-weight linear phosphates that appear to function as phosphate source for the synthesis of nucleic acids and phospholipids or as a source of energy for ATP synthesis (63, 86). The appearance of polyphosphate bodies in transmission electron microscopy varies considerably with the fixation and poststaining procedures, from electron-dense, to porous, to structures with an electron-transparent center and an electron-dense periphery (71).

The identity of phosphorus-containing storage seen by transmission electron microscopy was confirmed by X-ray microanalysis, where phosphorous interaction with electrons was shown by X-ray emission of characteristic energy and wavelength (72).

In situ X-ray dispersive analysis also demonstrated the sequestering of metals into polyphosphate bodies (67), suggesting that the bodies may serve as storage sites for metal ions and for detoxification if metals are present at toxic levels. Stereological techniques for electron microscope morphometry have been used to quantify changes in *Plectonema boryanum* after heavy metal exposure; these studies showed that Cd exposure caused a significant increase in the volume but not number of polyphosphate bodies (101). These investigators proposed that polyphosphate bodies are a means of binding cations in a relatively nontoxic state within the cell, useful for binding and detoxification of metals after they have entered the cell.

NUTRIENT STARVATION AND REPLETION The major ultrastructural change in phosphorus-deficient cells is the loss of polyphosphate bodies (70, 84). Excess phosphate favors the formation of polyphosphate bodies (65), and the "polyphosphate over plus" phenomenon, or storage of polymer when phosphate is added to a phosphate-deficient culture, has been observed in batch culture (84, 109) as well as in phosphate-limited continuous culture (59). Phosphorus uptake is very rapid upon addition of phosphorus to phosphorus-deficient cells of *Anabaena variabilis* (64) and of *Synechococcus* (78). *A. variabilis* takes up and stores phosphorus as it becomes more phosphorus-sufficient in excess of the phosphorus content required to support the cell's maximum growth rate. Ten percent of the total cell phosphorus in *Synechococcus* was present as acid-insoluble polyphosphate, which did not turn over during balanced exponential growth (59).

Labeled phosphate given to phosphorus-deficient *Synechococcus* appears first in an acid-soluble pool, moving rapidly into accumulated polyphosphate, which accounts for 60% of the total in cell (59); polyphosphate was broken down after eight hours, even in the presence of excess external orthophosphate. Phosphorus was transferred into other cell fractions such as RNA. Polyphos-

phate accumulation and body formation is likely mediated by polyphosphate synthetase—its activity increased at least 20-fold during phosphate starvation or in phosphate-limited growth. Polyphosphatase activity changed little in different growth conditions (59), suggesting that breakdown of polyphosphate is regulated by modulation of existing enzyme activity.

Polyphosphate-deficient mutants of *Synechococcus* have been described after mutagenesis, sulfur deficiency, and penicillin enrichment (130); mutant cells had altered levels of polyphosphate kinase and no granules. Other enzyme activities and their regulation should be studied in these mutants so the physiological role of polyphosphates under different environmental conditions can be determined.

Polyphosphate granules are elaborated in some species, but not in others, upon sulfur starvation (78, 79); their mean size and number increase with both degree and duration of sulfur deficiency in those species where they accumulate. Polyphosphate pools rise when *Synechoccus* is starved for nitrogen (59).

Polyglucose Bodies

Polyglucose bodies or glycogen bodies (α-granules; 28) are discrete granules or rods located between the thylakoids. By staining (53) or isolation (28) they have been shown to consist of an intracellular polysaccharide with many of the features of a carbon and energy reserve. When hydrolyzed with acid, D-glucose is formed; α-amylase treatment yielded maltose and an α-macrodextrin that accounted for 11–14% of the polysaccharide (28, 114). In exponential growth phase, photoautotrophically grown batch cultures contained 10–20% glycogen by dry weight (6, 80), whereas glycogen can account for 40–60% of the dry weight of cultures starved for nitrogen (6, 80, 120, 132) but having an excess of a utilizable carbon source.

Addition of combined nitrogen to starved cultures results in a large decrease in glycogen content before cell division commences (6, 120). Starvation and restoration of iron causes the same increase and decrease in polyglucose granules (106); the initial event in reconstitution was the utilization of the granules. Photosynthetic starvation conditions, such as removal of light or CO_2 or the addition of DCMU (a specific inhibitor of photosystem II) to the medium, cause a mobilization of polyglucose, suggesting the utilization of α-glucan as a reserve carbon for the cell (80).

The reserve function of the intracellular polysaccharide is shown by the ability of cells to return to normal growth at its expense. Simultaneous addition of a nitrogen source to, and removal of CO_2 from, nitrogen-starved cells showed *Synechococcus* could use polyglucose as an endogenous carbon source. Polysaccharide turned over during periods of constant cellular polyglucose concentration as was shown by pulse-chase experiments (80).

Incubation in the dark under aerobic conditions results in a gradual decrease

in the amount of polyglucose in cells of *Synechocuccus* (80) and of *Oscillatoria* (93). Both in batch and continuous cultures with a light-dark cycle, polyglucose accumulated during the light phase and was utilized during the subsequent dark period (133). Mechanisms of regulating the mobilization of the endogenous reserves are unknown. Dark metabolism via the oxidative pentose phosphate cycle has most recently been reviewed by Smith (117).

Ultrastructural comparisons of three species of cyanobacteria were made after growth in photoautotrophic and photoheterotrophic conditions on glucose or ribose (10). The photoautotrophic strain did not vary in ultrastructure under the two conditions; the photoheterotrophic strain showed accumulation of polyglucose and cyanophycin granules and fewer ribosomes in ribose-grown cultures when compared to photoautotrophically or glucose-grown cells. *Chlorogloea fritschii,* able to grow chemoheterotrophically as well as photoautrophically or photoheterotrophically, seldom contained cyanophycin granules when grown photoheterotrophically, but contained numerous polyglucose bodies and PHB deposits under these conditions.

Higher polyglucose content has also been reported in other cyanobacteria grown heterotrophically (98, 100). Glucose is oxidized via the oxidative pentose cycle during heterotrophic growth (98); polyglucose synthesis is the main biosynthetic process in heterotrophically metabolizing cells. Typical autotrophs store polyglucose in the light and metabolize it in the dark (93, 133). Cells capable of anoxygenic photosynthesis possess two mechanisms of anaerobic polyglucose metabolism for dark energy formation: fermentation to lactic acid, or respiration with elementary sulfur as electron acceptor (93). These cells in particular show great metabolic flexibility.

Synechococcus grown in high white-light intensity showed many more polyglucose granules than cells grown in red or low white light (37), but a decrease in granules was observed with increasing light intensity in *Spirulina platensis* (131) and *Oscillatoria chalybia* (53).

Gas Vacuoles

Many planktonic cyanobacteria possess gas vacuoles—organelles made up of assemblages of hollow cylindrical structures called gas vesicles; reviews of their structure and function have been published (135, 136, 139). The density of the gas vesicle is about 0.12 gcm^{-2} (140), so if enough vesicles are in a cell it can become buoyant.

Walsby's studies on planktonic cyanobacteria in culture have shown that cells select a position of optimum irradiance in a vertically decreasing light irradiance gradient by means of regulation of gas vacuolation in response to light irradiance. Loss of buoyancy due to collapse of gas vesicles by rising turgor pressure (driven by photosynthesis) occurs upon transfer of cells to high irradiance (141). *Trichodesmium thieboutii,* living in the ocean with high

hydrostatic pressure, has very strong gas vesicles that do not collapse by turgor pressure (138).

Absence of a single gas vacuole protein in a spontaneous, nonreverting mutant of *Anabaena flos-aquae* has been demonstrated (137). The single protein (139) is one of the 15 most abundant proteins in the wild-type cell and has a molecular weight of about 20 kd (9). Although it was speculated to be a plasmid-specified protein, only negative evidence for this hypothesis is available. The majority of planktonic cyanobacteria constitutively produce gas vacuoles; recently, changes in culture conditions that stimulate the induction of de novo synthesis of gas vacuoles in hormogonia in *Nostoc muscorum* were described (9). Changes in the culture conditions such as medium dilution, removal of $NaNO_3$ or another salt, or increased light intensity all induce gas vacuole formation, but the gas vacuoles disappeared about three days after formation.

Because the induction of gas vacuoles is part of the differentiation process of hormogonia, vacuoles could provide the hormogonia with buoyancy to aid in their dispersal; loss of gas vacuoles and loss of buoyancy would bring the cells to a new ecological niche (9). Increase in light intensity has been shown to lead to an increase in gas vesicle concentration in *Spirulina platensis* (131).

Poly-β-Hydroxybutyrate

Little is known about the lipid reserves of cyanobacteria. Poly-β-hydroxybutyrate (PHB), a polymer unique to bacteria, has been observed in only a few cyanobacteria ultrastructurally (19, 68) and biochemically (19, 24). The slightly electron-dense granules, generally spherical in shape, are surrounded by a single membrane. For production of PHB in *Chlorogloeopsis fritschii* it was necessary in one case to supplement the medium with sodium acetate (24), as is true in *Aphanocapsa* 6308 (Allen, unpublished data); however, in other hands, PHB could be extracted from control cells and observed in the electron microscope (69).

PHB accumulated without the addition of reduced carbon compounds in *Spirulina platensis* (19). In this strain PHB accumulation increased during exponential growth, reached a maximum as the cell entered stationary phase, and then decreased. A maximum of 6% total dry weight PHB was recoverable from *S. platensis* either with or without sodium acetate in the medium. No PHB was observed, however, in the anoxygenic photosynthesizer *Oscillatoria limnetica* under any condition, even grown in the presence of acetate (93), consistent with its relative insignificance as a storage material.

Inclusions of Infrequent Occurrence

A large number of other inclusions have been seen in some species of cyanobacteria (reviewed in 43, 66), but they are common to only certain isolates.

These include cylindrical bodies, trilamellar bodies, microplate-microtubule arrays, microfilaments, microtubule-like arrays, paracrystalline inclusions, spherical bodies, lamellasomes, spherical spoke inclusions, and peripheral lamellar sheets. The function and composition of none of these is known. Jensen has proposed that some of the inclusions might be plasmid-coded (43); however, much work other than observation must be done on these infrequently observed inclusions before more can be said.

CONCLUDING REMARKS

Nutrient starvation and repletion have great effects on the occurrence and amount of the various cyanobacterial cellular inclusions. Much information has been gained in the last ten years on their structure, chemistry, and function, but much remains to be learned both from laboratory and field studies. Many structures have been identified in some species and under some growth conditions, but little more is known about them; careful physiological studies must be carried out along with their isolation and characterization. Studies of effects of the environment on even well-characterized inclusions show species variation; work must be done on more species under identical conditions in order to determine whether differences are due to species variation or experimental variation.

The role of cyanobacterial reserve materials such as cyanophycin, polyphosphate, and polyglucose has recently been reassessed; the hypothesis has been presented that they have a role in the ordered assimilation of nitrogen, phosphate, and carbon (25). The incorporation of either ^{15}N-ammonium salts, ^{14}C-bicarbonate, or ^{32}P-phosphate show a preferential flow of isotope, in early time samples, into "reserve" polymers prior to distribution into macromolecules necessary for cell growth. The immediate synthesis of cyanophycin when nitrogen is added to nitrogen-depleted *Aphanocapsa*—before phycocyanin or other protein is synthesized—has been observed (3). Labeled phosphate taken into phosphorus-deficient *Synechococcus* appeared first in an acid-soluble pool, but then moved rapidly into polyphosphate that accumulated (59).

These data suggest a possible role for each of these polymers as dynamic reservoirs in nutrient assimilation, with great relevance for cells in certain environmental conditions where nutrients are limiting. A more direct role for cyanophycin in nitrogen assimilation, perhaps as a reservoir between nitrogen fixation and export of glutamine from heterocysts into vegetative cells, has also been suggested from data showing similar amounts of cyanophycin in heterocysts and vegetative cells as well as high activity of cyanophycin-metabolizing enzymes in heterocysts (61). Much more research is necessary to assess whether many of the inclusions are only reserve polymers or whether they have other metabolic roles within the cell.

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Literature Cited

1. Alberte, R. S., Tel-Or, E., Packer, L., Thornber, J. P. 1980. *Nature* 284:481–83
2. Allen, M. M., Hawley, M. A. 1983. *J. Bacteriol.* 154:1480–84
3. Allen, M. M., Hutchison, F. 1980. *Arch. Microbiol.* 128:1–7
4. Allen, M. M., Hutchison, F., Weathers, P. J. 1980. *J. Bacteriol.* 141:687–93
5. Allen, M. M., Morris, R., Zimmerman, W. 1984. *Arch. Microbiol.* In press
6. Allen, M. M., Smith, A. J. 1969. *Arch. Microbiol.* 69:114–20
7. Allen, M. M., Weathers, P. J. 1980. *J. Bacteriol.* 141:959–62
8. Andrews, T. J., Abel, K. M. 1981. *J. Biol. Chem.* 356:8445–51
9. Armstrong, R. E., Hayes, P. K., Walsby, A. E. 1983. *J. Gen. Microbiol.* 128:263–70
10. Baulina, O. I., Mineeva, L. A., Suleimanova, Sh. S., Gusev, M. V. 1981. *Mikrobiologiya* 50:523–27
11. Bennett, A., Bogorad, L. 1973. *J. Cell Biol.* 58:419–35
12. Beudeker, R. F., Codd, G. A., Kuenen, J. G. 1981. *Arch. Microbiol.* 129:185–89
13. Beudeker, R. F., Kuenen, J. G. 1981. *FEBS Lett.* 131:269–74
14. Bogorad, L. 1975. *Ann. Rev. Plant Physiol.* 26:369–401
15. Boussiba, S., Richmond, E. 1980. *Arch. Microbiol.* 125:143–47
16. Bryant, D. A., Cohen-Bazire, G. 1981. *Eur. J. Biochem.* 119:415–24
17. Bryant, D. A., Guglielmi, G., Tandeau de Marsac, N., Castets, A. M., Cohen-Bazire, G. 1979. *Arch. Microbiol.* 123: 113–27
18. Bryceson, I., Fay, F. 1981. *Marine Biol.* 61:159–66
19. Campbell, J. III, Stevens, S. E. Jr., Balkwell, D. L. 1982. *J. Bacteriol.* 149:361–63
20. Canaani, O., Gantt, E. 1982. *Proc. Natl. Acad. Sci. USA* 79:5277–81
21. Canaani, O., Lipschultz, C. A., Gantt, E. 1980. *FEBS Lett.* 115:225–29
22. Cannon, G. C., Shively, J. M. 1983. *Arch. Microbiol.* 134:52–59
23. Carpenter, E. J., Price, C. C. 1976. *Science* 191:1278–80
24. Carr, N. G. 1966. *Biochim. Biophys. Acta* 120:308–10
25. Carr, N. G., Gupta, M., Rylah, J., McKie, J. 1982. *Abstr. IV Int. Symp. Photosynth. Prokaryotes,* p. B12. Bombannes, Fr.
26. Carr, N. G., Whitton, B. A., eds. 1973. *The Biology of Blue-green Algae.* Berkeley: Univ. Calif. Press. 676 pp.
27. Carr, N. G., Whitton, B. A., eds. 1982. *The Biology of Cyanobacteria.* Berkeley: Univ. Calif. Press. 688 pp.
28. Chao, L., Bowen, C. C. 1971. *J. Bacteriol.* 105:331–38
29. Codd, G. A., Cook, C. M., Stewart, W. D. P. 1979. *FEMS Microbiol. Lett.* 6:81–86
30. Codd, G. A., Stewart, W. D. P., 1976. *Planta* 130:323–26
31. Codd, G. A., Stewart, W. D. P. 1979. *Arch. Microbiol.* 113:105–110
32. Cohen-Bazire, G., Bryant, D. A. 1982. See Ref. 27, pp. 143–90
33. Coleman, J. R., Seeman, J. R., Berry, J. A. 1982. *Carnegie Inst. Wash. Year Book 81.* pp. 83–87

34. Cox, G., Benson, D., Dwarte, D. M. 1981. *Arch. Microbiol.* 130:165–74
35. de Vasconcellos, L., Fay, P. 1974. *Arch. Microbiol.* 96:271–79
36. Döhler, G. 1976. *Planta* 131:129–33
37. Döhler, G., Barckhausen, R., Ruppel, M. 1981. *Z. Naturforsch.* 36c:907–9
38. Doolittle, W. F. 1979. *Adv. Microb. Physiol.* 20:1–102
39. Drews, G., Weckesser, J. 1982. See Ref. 27, pp. 333–58
40. Eley, J. H. 1971. *Plant and Cell Physiol.* 12:311–16
41. Evans, E. H., Foulds, I., Carr, N. G. 1976. *J. Gen. Microbiol.* 92:147–55
42. Fleming, H., Haselkorn, R. 1974. *Cell* 3:159–70
43. Fliesser, S. B., Jensen, T. E. 1982. *Cytobios* 33:203–22
44. Fogg, G. E., Stewart, W. D. P., Fay, P., Walsby, A. E. 1973. *The Blue-green Algae.* London: Academic. 459 pp.
45. Foulds, I., Carr, N. G. 1977. *FEMS Lett.* 2:117–19
46. Gantt, E. 1977. *Photochem. Photobiol.* 26:685–89
47. Gantt, E. 1980. *Int. Rev. Cytol.* 66:45–80
48. Gantt, E. 1981. *Ann. Rev. Plant Physiol.* 32:327–47
49. Gantt, E., Lipschultz, G. A., Grabowski, J., Zimmerman, B. K. 1979. *Plant Physiol.* 63:615–20
50. Gendel, S., Ohad, I., Bogorad, L. 1979. *Plant Physiol.* 64:786–90
51. Giddings, T. H., Staehlin, L. A. 1979. *Biochim. Biophys. Acta* 546:373–82
52. Giddings, T. H. Jr., Wasmann, C., Staehlin, L. A. 1983. *Plant Physiol.* 71:409–19
53. Giesy, R. M. 1964. *Amer. J. Bot.* 51:388–96
54. Gingrich, J. C., Blaha, L. R., Glazer, A. N. 1982. *J. Cell Biol.* 92:261–68
55. Glazer, A. N. 1976. *Photochem. Photobiol. Rev.* 1:71–115
56. Glazer, A. N. 1982. *Ann. Rev. Microbiol.* 36:173–98
57. Goedheer, J. C. 1976. *Photosynthetica* 10:411–22
58. Golecki, J. R., Drews, G. 1982. See Ref. 27, pp. 125–42
59. Grillo, J. F., Gibson, J. 1979. *J. Bacteriol.* 140:508–17
60. Guglielmi, G., Cohen-Bazire, G., Bryant, D. A. 1981. *Arch. Microbiol.* 129:181–89
61. Gupta, M., Carr, N. G. 1981. *J. Gen. Microbiol.* 125:12–23
62. Hällgren, J. E., Huss, K. 1975. *Physiol. Plant.* 34:171–76
63. Harold, F. M. 1966. *Bacteriol. Rev.* 30:772–94
64. Healey, F. P. 1982. See Ref. 27, pp. 105–24
65. Jensen, T. E. 1969. *Arch. Mikrobiol.* 67:328–38
66. Jensen, T. E. 1984. *Cytobios* 39:35–62
67. Jensen, T. E., Baxter, M., Rachlin, W., Jani, V. 1982. *Environ. Pollution (Ser. A)* 27:119–27
68. Jensen, T. E., Sicko, L. M. 1971. *J. Bacteriol.* 106:683–86
69. Jensen, T. E., Sicko, L. M. 1971. *Cytologia* 38:381–91
70. Jensen, T. E., Sicko, L. M. 1974. *Can. J. Microbiol.* 9:1235–39
71. Jensen, T. E., Sicko-Goad, L., Ayala, R. P. 1977. *Cytologia* 42:357–69
72. Kessel, M. 1977. *J. Bacteriol.* 129: 1502–5
73. Kumar, A., Tabita, F. R., Van Baalen, C. 1982. *Arch. Microbiol.* 133:103–9
74. Lanaras, T., Codd, G. A. 1981. *Planta* 153:279–85
75. Lang, N. 1968. *Ann. Rev. Microbiol.* 22:15–46
76. Lang, N., Simon, R. D., Wolk, C. P. 1972. *Arch. Microbiol.* 83:313–20
77. Lau, R. H., MacKenzie, M. M., Doolittle, W. F. 1977. *J. Bacteriol.* 132:771–78
78. Lawry, N. H., Jensen, T. E. 1979. *Arch. Microbiol.* 120:1–7
79. Lawry, N. H., Simon, R. D. 1982. *J. Phycol.* 18:391–99
80. Lehmann, M., Wöber, G. 1976. *Arch. Microbiol.* 111:93–97
81. Lemasson, C., Tandeau de Marsac, N., Cohen-Bazire, G. 1973. *Proc. Natl. Acad. Sci. USA* 70:3130–33
82. Lewin, R. A. 1981. In *The Prokaryotes,* Vol. I, ed. M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegl, pp. 257–66. Berlin: Springer. 1102 pp.
83. Lichtle, C., Thomas, J. C. 1976. *Phycologia* 15:393–404
84. Livingston, D., Whitton, B. A. 1983. *Br. Phycol. J.* 18:29–38
85. Lundell, D. J., Williams, R. C., Glazer, A. N. 1981. *J. Biol. Chem.* 256:3580–92
86. Merrick, J. M. 1979. In *The Photosynthetic Bacteria,* ed. R. K. Clayton, W. R. Sistrom, pp. 199–219. New York: Plenum. 946 pp.
87. Miller, L. S., Holt, S. C. 1977. *Arch. Microbiol.* 115:185–98
88. Nierzwicki-Bauer, S. A., Balkwill, D. L., Stevens, S. E. Jr. 1983. *J. Microsc.* In press
89. Nierzwicki-Bauer, S. A., Balkwill, D.

L., Stevens, S. E. Jr. 1982. *J. Ultrastruc. Res.* 84:73–82
90. Nierzwicki-Bauer, S. A., Balkwill, D. L. Stevens, S. E. Jr. 1983. *J. Cell Biol.* 97:713–22
91. Ohki, K., Gantt, E. 1983. *J. Phycol.* 19:359–64
92. Ohki, K., Katoh, T. 1976. *Planta* 129:249–51
93. Oren, A., Shilo, M. 1979. *Arch. Microbiol.* 122:77–84
94. Ownby, J. D., Shannahan, M., Hood, E. 1979. *J. Gen. Microbiol.* 110:255–261
95. Pankratz, H. S., Bowen, C. C. 1963. *Am. J. Bot.* 50:387–99
96. Paone, D. A. M., Stevens, S. E. Jr., 1981. *Plant Physiol.* 67:1097–1100
97. Peat, A., Whitton, B. A., 1968. *Arch. Microbiol.* 63:170–76
98. Pelroy, R. A., Kirk, M. R., Bassham, J. A. 1976. *J. Bacteriol.* 128:623–32
99. Peterson, R. B., Dolan, E., Calvert, H. E., Ke, B. 1981. *Biochim. Biophys Acta* 634:237–48
100. Raboy, B., Padan, E., Shilo, M. 1976. *Arch. Microbiol.* 110:77–85
101. Rachlin, J. W., Jensen, T. E., Baxter, M., Jani, V. 1982. *Arch. Environ. Contam. Toxicol.* 11:323–33
102. Rippka, R., Waterbury, J. B., Cohen-Bazire, G. 1974. *Arch. Microbiol.* 100:419–36
103. Rodgers, G. A., Stewart, W. D. P. 1977. *New Phytol.* 78:441–58
104. Rosinski, J., Hainfeld, J. F., Rigbi, M., Siegelman, H. W. 1981. *Ann. Bot. Lond.* 47:1–12
105. Sharma, P., Bergman, B., Hällbom, L., v. Hofsten, A. 1982. *New Phytol.* 92: 573–79
106. Sherman, D. M., Sherman, L. A. 1983. *J. Bacteriol.* 156:393–401
107. Shively, J. M. 1974. *Ann. Rev. Microbiol.* 28:167–87
108. Shively, J. M., Ball, F. L., 1973. Brown, D. H., Saunders, R. E. *Science* 182:584–86
109. Sicko-Goad, L., Jensen, T. E. 1976. *Am. J. Bot.* 63:183–88
110. Siegelman, H. W., Kycia, J. H., 1982. *Plant Physiol.* 70:887–97
111. Simon, R. D. 1971. *Proc. Natl. Acad. Sci. USA* 68:265–67
112. Simon, R. D. 1973. *J. Bacteriol.* 114:1213–16
113. Simon, R. D. 1973. *Arch. Microbiol.* 92:115–22
114. Simon, R. D. 1976. *Biochim. Biophys. Acta* 422:407–18
115. Simon, R. D., Lawry, N. H., McLendon, G. L. 1980. *Biochim. Biophys. Acta* 626:277–81
116. Simon, R. D., Weathers, P. 1976. *Biochim. Biophys. Acta* 420:165–76
117. Smith, A. J. 1982. See Ref. 27, pp. 47–85
118. Stanier, R. Y., Cohen-Bazire, G. 1977. *Ann. Rev. Microbiol.* 31:225–74
119. Stevens, C. L. R., Myers, J. 1976. *J. Phycol.* 12:99–105
120. Stevens, S. E. Jr., Balkwill, D. L., Paone, D. A. M. 1981. *Arch. Microbiol.* 130:204–12
121. Stevens, S. E. Jr., Paone, D. A. M., Balkwill, D. L. 1981. *Plant Physiol.* 67:716–19
122. Stewart, W. D. P. 1977. *Br. Phycol. J.* 12:89–115
123. Stewart, W. D. P., Codd, G. A. 1975. *Br. Phycol. J.* 10:273–78
124. Sutherland, J. M., Herdman, M., Stewart, W. D. P. 1979. *J. Gen. Microbiol.* 115:273–87
125. Tabita, F. R., Stevens, S. E. Jr., Gibson, J. L. 1976. *J. Bacteriol.* 125:531–39
126. Tandeau de Marsac, N. 1977. *J. Bacteriol.* 130:82–91
126a. Tandeau de Marsac, N. 1983. *Bull. Inst. Past.* 81:201–54
127. Tandeau de Marsac, N., Cohen-Bazire, G. 1977. *Proc. Natl. Acad. Sci. USA* 74:1635–39
128. Taylor, M. M., Storck, R. 1964. *Proc. Natl. Acad. Sci. USA* 52:958–65
129. Tempest, D. W., Neijssel, O. M., Zavenboom, W. 1983. *Soc. Gen. Microb. Symp.* 34:119–152
130. Vaillancourt, S., Beauchemin-Newhouse, N., Cedergren, R. J. 1978. *Can. J. Microbiol.* 24:112–16
131. van Eykelenburg, C. 1979. *Antonie van Leeuwenhoek* 45:369–390
132. van Eykelenburg, C. 1980. *Antonie van Leeuwenhoek* 46:113–27
133. van Liere, L., Mur, L. R., Gibson, C. E., Herdman, M. 1979. *Arch. Microbiol.* 123:315–18
134. van Liere, L., Zevenboom, M., Mur, L. R. 1977. *Prog. Wat. Tech.* 8:301–12
135. Walsby, A. E. 1973. See Ref. 26, pp. 340–52
136. Walsby, A. E. 1975. *Ann. Rev. Plant Physiol.* 26:427–39
137. Walsby, A. E. 1977. *Arch. Microbiol.* 114:167–70
138. Walsby, A. E. 1978. *Br. Phycol. J.* 13:103–16
139. Walsby, A. E. 1978. *Symp. Soc. Gen. Microbiol.* 28:327–58
140. Walsby, A. E., Armstrong, R. E. 1979. *J. Mol. Biol.* 129:279–58
141. Walsby, A. E., Booker, M. J. 1980. *Br. Phycol. J.* 15:311–19

142. Wang, R. T., Stevens, C. L. R., Myers, J. 1977. *Photochem. Photobiol.* 25:103–8
143. Weathers, P. J., Chee, H. L., Allen, M. M. 1980. *Arch. Microbiol.* 118:1–6
144. Weber, M., Wöber, G. 1975. *Carboyhydrate Res.* 39:295–302
145. Williams, R. C., Gingrich, J. C., Glazer, A. N. 1980. *J. Cell Biol.* 85:558–66
146. Wood, N. B., Haselkorn, R. 1979. In *Limited Proteolysis in Microorganisms*, ed. G. N. Cohen, H. Holzer, pp. 159–66. Bethesda, MD: U.S. DHEW Publ. No. (NH) 79–1591. 255 pp.
147. Wood, N. B., Haselkorn, R. 1980. *J. Bacteriol.* 141:1375–85
148. Yamanaka, G., Glazer, A. N. 1980. *Arch. Microbiol.* 124:39–47
149. Yamanaka, G., Glazer, A. N. 1981. *Arch. Microbiol.* 130:23–30
150. Yamanaka, G., Glazer, A. N., Williams, R. C. 1980. *J. Biol. Chem.* 255:11004–10
151. Zilinskas, B. A., Howell, D. A. 1983. *Plant Physiol.* 71:379–87

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THE ROLE OF OXYGEN AND ITS DERIVATIVES IN MICROBIAL PATHOGENESIS AND HOST DEFENSE

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INTRODUCTION

Mononuclear phagocytes and granulocytes readily kill many potentially infectious agents, even in a nonimmune individual. In this review we discuss the mechanisms of phagocyte-induced killing of microorganisms, with particular emphasis on the role of the microbicidal metabolites superoxide anion (O_2^-),

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hydroxyl radical (OH^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). Determining how certain organisms avoid being killed by either granulocytes or mononuclear phagocytes is essential in understanding both the oxygen-dependent and -independent microbicidal mechanisms of "professional" phagocytes.

The virulence of several microorganisms clearly depends upon either their innate resistance to the reactive products of oxidative metabolism, their production of superoxide dismutase and catalase, which neutralize these toxic metabolites, or the failure of the microorganism to induce an oxidative metabolic burst that enables them to evade oxygen-dependent killing by phagocytic cells.

Once an infectious agent is phagocytized by either monocytes, macrophages, or polymorphonuclear leukocytes, it may either: be killed and digested; remain viable and replicate; or persist within the cell. Additionally, antigens of the organism may induce a humoral and cellular immune response.

A variety of factors influence the fate of these ingested microorganisms. Thus, the route of exposure of the microorganism to the host, the metabolic state of the host's phagocytic cells, the types, concentrations, and availability of lysosomal components within the "professional" phagocytes, the presence of nonspecific factors such as complement, the presence of specific opsonic antibodies, the overall state of the host, and the presence of specific virulence factors produced by the microbial invader all determine the ultimate outcome of the host-parasite interaction.

MECHANISMS OF MICROBICIDAL ACTIVITY OF POLYMORPHONUCLEAR PHAGOCYTES

Lysosomes and Oxygen-Independent Microbicidal Mechanisms

The initial encounter of microorganisms with polymorphonuclear phagocytes results in binding of the organism to the phagocyte surface membrane and invagination of the phagocyte membrane into a phagosome. This interaction triggers both the generation of microbicidal metabolites, via what is termed the respiratory burst, and the fusion of lysosomes with the plasma membrane of the phagosomes, resulting in the release of the lysosomal contents. This latter process is called degranulation in polymorphonuclear phagocytes.

Polymorphonuclear phagocytes in humans have two types of granules or lysosomes containing microbicidal and hydrolytic digestive enzymes. The primary or azurophilic granules contain myeloperoxidase, a number of acid hydrolases, lysozyme, neutral proteases, and cationic antibacterial proteins (6). Specific granules contain lactoferrin, lysozyme, B_{12}- binding protein, and phospholipase A. The specific granules have been shown to fuse with the plasma membrane prior to fusion of the membrane with the primary granules (5).

Except for myeloperoxidase, which will be discussed later, the role of the granule contents in the killing of microorganisms is not as clearly defined as the microbicidal activity of the toxic oxygen metabolites. Although aerobic killing is more efficient, polymorphonuclear phagocytes can kill *Salmonella typhimurium* (110), *Candida parapsilosis*, and *C. pseudotropicalis* (70) as well as several other bacterial species under anaerobic conditions (76).

CATIONIC PROTEINS Hirsch first isolated bactericidal substances from polymorphonuclear leukocyte granules (48, 49), which were later shown to be a complex of cationic proteins (124). Antibacterial cationic proteins (124) and antifungal cationic proteins have been isolated from human granulocytes and monocytes (36, 74).

Large-molecular-weight cationic proteins have been shown to inhibit multiplication and increase the outer membrane permeability of strains of *E. coli*, *S. typhimurium*, and *Pseudomonas aeruginosa* (38), but did not affect either most gram-positive bacterial species, *Proteus vulgaris*, *Serratia marcescens*, or *Candida* strains. Lower-molecular-weight (10,000–25,000 daltons) cationic proteins inhibit the multiplication of staphylococci (99). In vivo, cationic proteins are probably more efficient in killing microorganisms when acting with other antimicrobial mechanisms of polymorphonuclear phagocytes.

LACTOFERRIN The iron-binding protein lactoferrin may bind the iron in the growth medium, thus inhibiting growth of microorganisms (25, 79, 91). Saturation of this iron-binding protein with ferritin or other iron-containing compounds reduces the inhibitory effect of phagocytes on growth of *Pseudomonas* (24) and *C. albicans* (60). However, granulocytes lacking lactoferrin are still able to kill *S. aureus* (109), and depletion of specific granules does not eliminate the bactericidal capacity of polymorphonuclear leukocytes (118).

LYSOZYME, ACID HYDROLASES, AND NEUTRAL PROTEASES Lysozyme is found in both primary and specific granules. The enzyme acts on the ß 1–4 glycosidic linkage between N-acetyl muramic acid and N-acetyl glucosamine in the murein backbone of the peptidoglycan in bacterial cell walls. The cell walls of the majority of bacteria involved in human diseases are resistant to the lytic action of lysozyme (99). Thus it is not clear that lysozyme alone has bactericidal activity.

Lysozyme may be able to kill bacteria whose resistance has been reduced by other bacteriostatic mechanisms of the leukocyte. Thus, *S. aureus* after treatment with penicillin may be killed by a combination of neutral proteases and lysozyme (37). All of these enzymes, however, probably play a more important role in digestion of microorganisms killed by other microbicidal mechanisms.

Oxygen-Dependent Microbicidal Mechanisms

RESPIRATORY BURST It has long been known that phagocytosis of microorganisms by granulocytes is accompanied by a dramatic increase in oxygen consumption (7), which is not cytochrome-linked (105). Sbarra & Karnovsky also found that there is an increase in oxidation of glucose through the hexose monophosphate shunt following phagocytosis (105). Oxygen metabolites such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (53) were found to be produced in greater amounts when phagocytes interacted with microorganisms. These changes, called the respiratory burst, can be measured by chemiluminescence, which is the generation of measurable light (1) and the increased reduction of nitroblue tetrazolium (yellow dye) to formazan (black precipitate) (106).

Iyer et al (53) were the first to state that the respiratory burst following phagocytosis might be related to the microbicidal activity of phagocytes. They felt that part of the oxygen consumed in the respiratory burst was converted to H_2O_2, which was used as a bactericidal agent by the phagocyte (53).

Babior et al first proposed that superoxide generated during the respiratory burst was a bactericidal agent in leucocytes (4). The interaction of oxygen with various enzymes and the intermediates formed has been outlined in several reviews (2, 64, 99). When the phagocyte membrane is stimulated by phagocytosis of microorganisms or by membrane-stimulating agents such as phorbol myristate acetate, oxygen is reduced to superoxide (4). This reaction is catalyzed by an oxidase probably associated with the phagocyte membrane (43).

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced in the hexose monophate shunt (HMS) is used as a substrate. Superoxide is further reduced by superoxide dismutase (SOD) or spontaneous dismutation to form H_2O_2. Hydrogen peroxide is reduced by catalase to O_2 and H_20.

$$2O_2 + NADPH \xrightarrow{\text{Oxidase}} 2O_2^- + NADP^+ + H^+ \quad 1.$$

$$2O_2^- + 2H^- \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad 2.$$

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \quad 3.$$

The first reaction occurs at the surface of the phagocyte or on the surface membrane invaginated into the phagosome (64). Within the phagosome, spontaneous dismutation of O_2^- to H_2O_2 can occur. Superoxide dismutase has been found in the cytosol of polymorphonuclear phagocytes, which can also act on O_2^-. However, it is not known if SOD is secreted into the phagocytic vacuole (63).

When the phagosome containing the microorganism fuses with lysosomes or granules, myeloperoxidase (MPO) is released into the vacuole. Hydrogen peroxide in the presence of halide ions and MPO produces microbicidal reactions due to the halogenation of proteins (61), which leads to aldehyde formation (112) and peptide cleavage (107).

Hydrogen peroxide can be reduced either by catalase, as shown in Reaction 3, or H_2O_2 can oxidize glutathione (GSH), as catalyzed by glutathione peroxidase (GSH-PO) to form H_2O.

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-PO}} \text{oxidized Glutathione} + 2H_2O \quad 4.$$

$$\text{Oxidized glutathione} + NADPH \rightarrow 2GSH + NADP^+ \quad 5.$$

MICROBICIDAL MECHANISMS The relative importance of oxygen metabolites in the microbicidal activity of polymorphonuclar phagocytes is largely based on studies demonstrating that phagocytes with genetic disorders in the respiratory burst mechanism cannot kill a large variety of microorganisms. These phagocyte-deficiency diseases will be discussed later. Although some organisms can be killed under anaerobic conditions, killing of most microorganisms is more efficient under aerobic conditions (76, 83).

It has been shown that the products of the respiratory burst such as superoxide and hydrogen peroxide are probably not involved in the production of energy for maintenance of the phagocytic cell (105). Thus, their only known function appears to be involved in the microbicidal activities of phagocytes.

There are three reactive forms of oxygen formed during the respiratory burst that may have microbicidal activity: superoxide anion (4), singlet oxygen ($^1O_2$) (1), and hydroxyl radicals (OH^-) (114). In cell-free superoxide generating systems, superoxide has less microbicidal activity for *E. coli* than H_2O_2 (3), and it was felt that the myeloperoxidase—H_2O_2—halide system was more efficient in killing bacteria (2, 63). However, the concentration of superoxide produced by the phagocytic cell in close contact with microorganisms is higher than that produced in the cell-free superoxide generating systems (3).

In one study, SOD, when added to cultures of leukocytes, inhibited bacterial killing (57), but these investigators felt that OH^- radicals were more efficient in killing bacteria. The presence of the unstable hydroxyl radical has been inferred from studies showing that either SOD or catalase will inhibit killing of some bacteria by cell-free superoxide generating systems (3), which indicated a product of superoxide and H_2O_2 was bactericidal for *Staphlococcus epidermidis*. Hydroxyl radicals are thought to be produced by the Haber-Weiss reaction, whereby H_2O_2 was produced by spontaneous dismutation of superoxide (2).

$$O_2^- + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2\ (^1O_2?) \quad 6.$$

The presence of hydroxyl radicals during phagocytosis in leukocytes has been detected by electron spin resonance spectroscopy (102), but its role in the microbicidal activities of neutrophils is not clear (99).

Singlet oxygen ($^1O_2$) is another reactive oxygen species whose activities have been deduced from indirect evidence. Allen et al felt that the light produced by cells during phagocytosis, which is detected by chemiluminescence, is due either to singlet oxygen reverting to O_2 to generate light or to singlet oxygen producing a light-emitting product (1). However, Babior suggested that superoxide or H_2O_2 may also cause chemiluminescence (2). Furthermore, singlet oxygen may be a product of the myeloperoxidase—halide—hydrogen peroxide system as shown by use of a singlet oxygen trapping reagent or singlet oxygen scavangers (101). Both serum components (80) and SOD may quench the activity of singlet oxygen (120).

After the phagocytic vacuoles containing microorganisms fuse with the azurophilic granules, myeloperoxidase is released and can catalyze the following reaction:

$$Cl^- + H_2O_2 \rightarrow ClO^- + H_2O \qquad 7.$$

Any of the halide ions (I^-, Br^-, Cl^-) can be oxidized (45, 61, 62), but it is more likely that Cl^- is used by the phagocyte because it is present in higher concentrations (8).

Hydrogen peroxide at a concentration of 0.5 mM killed 34% of an *E. coli* inoculum, but in the presence of leukocyte granule extracts, 99.9% of the *E. coli* were killed (84). Leukocyte granule extracts with 10 μM hydrogen peroxide killed some *E. coli* (9.9%), but hydrogen peroxide alone at this concentration did not kill any of the cells of *E. coli*. The myeloperoxidase-halide–hydrogen peroxide system is the most generally accepted microbicidal mechanism of neutrophils (2, 61, 64, 72).

There are several theories as to how microorganisms are damaged by products of the respiratory burst (99). After bacteria are incubated with MPO, hydrogen peroxide and radioactively labeled iodine, the bacterial cell wall becomes labeled (61). This iodination of bacteria was thought to be closely associated with the bactericidal effects of MPO and hydrogen peroxide. However, bacterial killing can occur even when iodination is blocked (81).

Another mechanism to explain bacterial death is that MPO, hydrogen peroxide, and halides decarboxylate amino acids from bacterial cell-wall protein chains, resulting in the cleavage of peptide bonds, the loss of cell-wall integrity, and the death of the bacteria (107). Also, bactericidal aldehydes may be generated from free amino acids by the MPO system (112).

The formation of hypochlorite by the MPO system can lead to the production of chloramines, which have been shown to oxidize sulfhydryl groups in *E. coli*,

resulting in the death of the bacteria (115, 116). Killing of *Streptococcus pneumoniae* was correlated with production of H_2O_2, and there was evidence that lipid peroxidation had occurred (108). The action of superoxide or singlet oxygen on bacterial cell walls could result in lipid peroxidation that, in turn, could inactivate enzymes or crosslink proteins.

MECHANISMS OF MICROBICIDAL ACTIVITY IN MACROPHAGES AND MONONUCLEAR PHAGOCYTES

Lysosomes

The lysosomes of resident peritoneal macrophages contain several hydrolases, which include acid phosphatase, ß-glucuronidase, and cathepsin D (28). When resident macrophages are activated by exposure to lymphokines or some bacteria (BCG or *Corynebacterium parvum)* these lysosomal enzyme levels increase in concentration (28). Lysozyme is thought to be secreted by macrophages (46), but the enzyme level does not increase within activated macrophages (28).

Because most pathogenic bacterial cell walls are protected against the effects of lysozyme, its direct role in phagocyte-induced killing is probably minimal, but once the murein backbone of pathogenic bacteria is exposed by other mechanisms, lysozyme may be able to digest the bacterial cell wall. Thus, the lysosomal hydrolases may function primarily to digest damaged microorganisms. Cationic proteins have been isolated from alveolar macrophages, which have a fungicidal activity (73).

There are also a number of ectoenzymes associated with macrophages that include alkaline phosphiodiesterase, nucleotidase, and amino-peptidase (59). Also, elastase, collagenase, and plasminogen activator are secreted by macrophages. These ectoenzymes may have some adverse affect on the microorganism's interaction with the macrophage. The presence of peroxidase in macrophages is controversial (50), and it is more readily detected in the immature monocytes of newly elicited peritoneal cells (59). The probable role of peroxidases in microbial killing will be discussed later.

There are significant differences in the enzyme levels in different types of mononuclear cells. For example, the highest concentration of lysozyme and hydrolases is found in alveolar macrophages (29), and alveolar macrophages are more dependent upon oxidative metabolism than are peritoneal macrophages, which depend more on glycolysis for energy production (92).

Oxygen-Dependent Microbicidal Mechanisms

Following phagocytosis, monocytes and macrophages undergo a respiratory burst similar to that described for polymorphonuclear phagocytes. The respiratory burst can be measured by increased oxygen consumption and hexose

monophosphate shunt activity (92, 111), superoxide generation (35, 56), hydrogen peroxide production (83, 89) and chemiluminescence (58) during phagocytosis by macrophages. The chemiluminescence generated by human monocytes can be reduced significantly by SOD (58, 90, 103) and the same concentration of SOD also reduces the killing of *Staphlococcus* by these human monocytes (103). In addition, human alveolar macrophages generate chemiluminescence following phagocytosis of heat-killed *S. aureus,* and SOD blocks this chemiluminescence generation (9). Superoxide has been shown to be produced by human monocytes (58). However, it is felt that the respiratory burst in monocytes is not as dramatic as that observed under similar conditions in polymorphonuclear phagocytes (9, 90, 103).

Activated macrophages respond to phagocytic stimulus with enhanced superoxide production, as compared to resident macrophages (56, 86, 104). There is a correlation between the killing of *Leishmania donovani,* (86), *Trypanosoma cruzi* (88), *Toxoplasma gondii* (87), and *Candida albicans* (104) by activated macrophages and the ability of the macrophage to produce O_2^- and H_2O_2 in the respiratory burst following phagocytosis.

The role of the myeloperoxidase-halide system in the microbicidal activities of macrophages is not clear. Peroxidase activity either has not been found or it has been found at very low levels in macrophages (50). Monocytes have been shown to iodinate *C. albicans;* however, agents that inhibit iodination do not inhibit killing of *C. albicans* (71), indicating halogenation is not required for killing.

It appears that when the monocyte or macrophage membrane is stimulated by phagocytosis, or by compounds such as phorbol myristate acetate, oxygen is reduced to superoxide in a reaction catalyzed by a membrane-associated oxidase utilizing NADPH (50). Superoxide may then be reduced to hydrogen peroxide by SOD. Hydroxyl radicals may be formed by the interaction of superoxide and hydrogen peroxide.

Catalase produced by microorganisms can be utilized by the phagocyte, or the catalase produced by the phagocyte can be transferred to the phagocytic vacuole to reduce hydrogen peroxide to oxygen and water. The production of oxygen metabolites by the phagocyte results in the death of the majority of phagocytized microorganisms. The determination of how some organisms are able to survive within mononuclear phagocytes should aid in the understanding of the mechanisms of phagocyte-induced killing.

DEFICIENCIES IN PHAGOCYTE FUNCTION

The role of oxidative metabolism in the microbicidal activities of polymorphonuclear phagocytes was first clearly demonstrated when it was found that patients with chronic granulomatous disease (CGD) had a defect in phagocyte function (52, 55, 96). The phagocytic respiratory burst characterized by increased oxygen consumption, hexose monophosphate activity, superoxide generation, and hydrogen peroxide formation is not seen in neutrophils and monocytes from CGD patients compared to cells from normal individuals (30, 52, 96, 97). CGD patients suffer from infections of the lung, liver, spleen, skin, and soft tissues with *Staphylococcus,* aerobic gram-negative rods, and opportunistic fungi (27, 55), which are catalase positive. However, these patients do not have repeated infections with catalase-negative organisms.

The production of hydrogen peroxide by catalase-negative organisms in the phagocytic vacuole apparently results in their death (78). Mutant strains of pneumococci or streptococci that do not produce hydrogen peroxide are not killed by CGD leukocytes (51, 94). These observations confirm the importance of hydrogen peroxide in the microbicidal activity of phagocytes.

Patients with myeloperoxidase (MPO) deficiencies do not suffer recurrent bacterial infections as frequently as do patients with chronic granultomatous disease (65). Phagocytes deficient in MPO respond to a phagocytic stimulus with a respiratory burst similar to normal phagocytes (65), including production of hydrogen peroxide (66) and superoxide (100). Killing or iodination of *Candida albicans* did not occur in MPO-deficient monocytes; however, MPO-deficient monocytes killed *Candida parapsilosis* as well as normal phagocytes (71). Because deficiencies in MPO have been found in significant numbers of hospitalized patients who do not show increased susceptibility to bacterial infections, the importance of the MPO-halide–hydrogen peroxide system, except perhaps in candidal infections, must be questioned (99).

The products of the respiratory burst such as superoxide and hydrogen peroxide may serve to kill microorganisms in MPO-deficient phagocytes. Furthermore, the assistance from nonoxidative mechanisms such as cationic proteins may be essential in the microbicidal activities of these phagocytic cells (71).

MICROBIAL DEFENSE AGAINST PHAGOCYTIC CELLS

Once a microbial cell breaches the physical defenses of the body (i.e. skin, mucous membrane, and so on) it encounters the phagocytic cells of the reticuloendothelial system. As described above, these host cells possess an extensive armamentarium designed to rid the body of these microbial invaders. However, pathogenic microorganisms have also developed a variety of mechanisms to avoid destruction by these phagocytes.

Different strains of most pathogenic microorganisms can differ widely in their capacity to evade host defenses and cause disease. A large variety of mechanisms that are beyond the scope of this discussion contribute to the varying degrees of virulence of microorganisms. However, since the products of oxidative metabolism in phagocytes appear to be a major component of the

microbicidal activity of these cells, pathogenic organisms must possess some mechanism whereby they circumvent the lethal effects of these products.

It is reasonable to suggest that the virulence of pathogens may be influenced by their ability to synthesize specific enzymes that destroy or neutralize the toxic products of oxidative metabolism. Indeed, because aerobic metabolism in all microorganisms results in the formation of these toxic, reactive products (i.e. O_2^-, $^1O_2$, $OH^\cdot$, and H_2O_2), aerobic and many anaerobic microorganisms synthesize one or more of the following enzymes: superoxide dismutase, catalase, and peroxidase. These enzymes function to catalyze the breakdown of the toxic substances produced by oxidative metabolism in order to form nontoxic end products.

Based upon this speculation, then, all microorganisms that synthesize these three enzymes should be more resistant to the killing mechanisms of phagocytes than those that do not. However, most microorganisms are killed by PMN phagocytes irrespective of their possessing these enzymes. In fact, there are relatively few species of bacteria that are not killed quite efficiently by the host's phagocytic cells. Therefore, it is unclear how the pathogenic microorganisms that are not killed by phagocytes avoid the lethal effects of oxidative metabolism.

Nocardia asteroides is a facultative intracellular pathogen that infects humans and a large variety of animals (11–13). It has been shown that the virulent strain, *N. asteroides* GUH-2, is not killed by either human monocytes or polymorphonuclear leukocytes even though this bacterium induces an oxidative metabolic burst in these phagocytes (40, 41). Furthermore, studies demonstrate that this organism is resistant to the superoxide anion generated in vitro, and it is very resistant to 1mM H_2O_2 (Beaman, unpublished observations; 39). It was observed that *N. asteroides* GUH-2 produces a unique superoxide dismutase (SOD) (22). This enzyme is composed of 4 subunits with a total molecular weight of 100,000 daltons. Unlike other bacterial SODs, the enzyme contains three metals—iron, manganese, and zinc—in equal molar ratios. Furthermore, the enzyme is selectively secreted into the medium during growth, and it becomes associated with the bacterial cell surface (22). In addition, the more virulent log phase cells of *N. asteroides* GUH-2 synthesize more SOD per cell than do the less virulent stationary phase cells of the same culture (22).

The role of this surface-associated superoxide dismutase in nocardial resistance to killing by PMN leukocytes obtained from humans was studied further by utilizing antibody prepared against purified nocardial SOD (Beaman, unpublished data). Single cell suspensions of *N. asteroides* GUH-2 were incubated for 30 minutes at 37°C in either normal rabbit IgG, rabbit antinocardial SOD IgG, or whole immune sera prepared against the nocardial cell (this latter sera had little or no detectable antibody against SOD). Log phase cells of

Listeria monocytogenes were used as a control for the microbicidal activity of the PMN leukocytes (119). The PMN leukocytes were able to kill more than 90% of the cells of *L. monocytogenes* in 2 hours, and antinocardial SOD IgG did not affect this listericidal activity. These PMN leukocytes did not kill any of the nocardial cells preincubated with either normal rabbit IgG or whole immune sera that lacked significant SOD antibody.

In sharp contrast, approximately 50% of the cells of *N. asteroides* GUH-2 preincubated with rabbit antinocardial SOD IgG were killed after 3 hours incubation. Pretreating PMN leukocytes with chlorpromazine inhibits the oxidative metabolic burst but not phagocytosis (40). Chlorpromazine-treated PMN leukocytes phagocytized the nocardial cells but there was a total elimination of the PMN leukocyte's ability to kill nocardial cells coated with rabbit antinocardial SOD IgG. Furthermore, diluting the anti–SOD IgG antibody by 1:5 prior to adding nocardial cells resulted in a concomitant loss in the susceptibility of these cells to killing by PMN leukocytes. Interestingly, chlorpromazine-treated PMN leukocytes were still able to kill approximately 70% of the cells of *L. monocytogenes*. These data indicated that the oxidative metabolic burst of PMN leukocytes was not the sole mechanism whereby they kill cells of *Listeria*.

The results described above indicate that the surface associated superoxide dismutase elaborated by the cells of *N. asteroides* GUH-2 appears to play a significant role in protecting these bacteria from the oxidative killing mechanisms of PMN phagocytes (Beaman, unpublished data). There is additional evidence that suggests that a direct correlation exists between the relative virulence of *N. asteroides*, its resistance to oxidative killing, and the amount, type, and location of SOD produced (22, 40, 41). *N. asteroides* 10905 strain is significantly less virulent for laboratory animals and it is more effectively killed by phagocytes than *N. asteroides* GUH-2 (11, 18, 19, 31–33). The 10905 strain has less SOD in its cytoplasm throughout its growth cycle than the GUH-2 strain. Furthermore, SOD appears not to be either secreted into the growth medium or become cell-surface associated in the less virulent strains of *Nocardia* (22).

As described above, superoxide dismutase catalyses the dismutation of the superoxide anion to form the more toxic hydrogen peroxide. Hydrogen peroxide alone or in combination with myeloperoxidase and halides is strongly microbicidal. Therefore, SOD would not be predicted to be the sole mechanism whereby microorganisms evade oxidative killing mechanisms. Catalase, which breaks down H_2O_2, must play a central role in this resistance to the products of oxygen metabolism.

Both the amount and properties of catalase of *Nocardia* were studied in our laboratory. The virulent *N. asteroides* GUH-2 synthesized large amounts of catalase within its cytoplasm during growth. Thus, the cells of a 48-hour

culture of *N. asteroides* GUH-2 (early stationary phase) grown in Brain Heart Infusion (BHI) broth had approximately 1482 units of catalase per mg protein within the cytoplasm. Cells of the less virulent *N. asteroides* 10905, at the same stage of growth in BHI, had 200 units per mg protein. In contrast, 18-hour cultures of *Escherichia coli* harvested at the same time and prepared in the same manner had only 22 units of catalase per mg protein, while 18-hour cells of *L. monocytogenes* possessed 120 units of catalase per mg protein (Beaman, unpublished data; 119). Both strains of *E. coli* and *L. monocytogenes* studied above were effectively killed (> 90% in 3 hours) by PMN leukocytes. At the same time *N. asteroides* GUH-2 was not killed (0% in 3 hours) and *N. asteroides* 10905 was intermediate in its susceptibility to PMN phagocytes (70% killing in 3 hours).

These data suggest that the high levels of catalase in the cytoplasm of *Nocardia* in combination with the secreted and surface-associated SOD play a role in both this organism's resistance to the microbicidal activity of PMN leukocytes and nocardial virulence (22, 39–41; Beaman, unpublished data).

The cells of virulent strains of *Mycobacterium tuberculosis* are not killed by phagocytic cells obtained from nonimmunized individuals; in contrast, avirulent organisms are more susceptible to the microbicidal activities of these phagocytes (54, 69, 123). A considerable amount of research has been done in an effort to define the factors relating to the virulence of *M. tuberculosis* as well as other mycobacteria (123). As with the biologically related nocardiae described above, the mechanisms whereby mycobacteria evade destruction by the host's phagocytes and then grow unimpeded within these cells is complex and not fully understood.

There appear to be multiple determinants related to the pathogenicity of *Mycobacterium* and *Nocardia,* and probably no component can be singled out as the agent responsible for either mycobacterial or nocardial virulence. Nevertheless, there appears to be a direct correlation between the amount of catalase produced by strains by *M. tuberculosis* and their relative virulence for both humans and laboratory animals (54, 69, 123). Furthermore, *M. tuberculosis* appears to synthesize an iron-containing superoxide dismutase that is different from the SOD produced by most of the nonpathogenic mycobacteria (69). As with *N. asteroides* GUH-2, *M tuberculosis* also secretes this SOD into the medium, and it probably becomes surface associated. In contrast, nonpathogenic mycobacteria appear not to secrete this enzyme, and the SOD remains localized within the cytoplasm of these nonpathogenic mycobacteria (69). Thus, the secreted and surface-associated SOD of these microorganisms may interact with and catalyze the dismutation of the O_2^- produced upon contact with either PMN leucocytes or mononuclear phagocytes. This would result in the production of H_2O_2 at the microbial surface, which could then be broken down by the high concentrations of intracellular catalase as the H_2O_2

penetrates the cell wall. It is conceivable that this sequence occuring at the cell surface may give the organism an advantage in resisting the microbicidal effects of these products of oxidative metabolism.

Jacket et al (54) studied a variety of strains and mutants of *M. tuberculosis* that varied in both their relative virulence for guinea pigs and their catalase content. Generally, those organisms that lacked catalase were susceptible to killing by H_2O_2 and were less virulent, while the strains that were highly virulent had large amounts of catalase and were resistant to H_2O_2. However, some strains that lacked catalase were still resistant to H_2O_2 and were virulent. Thus, there appeared to be some unidentifed mycobacterial component that acted independently of catalase and peroxidase activity in conferring resistance to H_2O_2 (54). It was suggested that the complex lipids in the mycobacterial cell envelope might play a significant role in both virulence and H_2O_2 resistance (54).

The cell walls of both *Mycobacterium* and *Nocardia* are structurally and chemically complex. They contain protein, peptidolipid, various complex lipids, glycolipids, mycolic acids, trehalose mycolates, the iron-binding mycobactins (or nocobactins), arabinogalactan mycolates, long-chain alcohols, and ketones in addition to the peptidoglycan. Many of these substances have been shown to be related to both the pathogenesis of these organisms as well as the induction of specific host responses (10, 13, 18, 19, 123).

In *Nocardia,* it was shown that the ultrastructural appearance and the chemical composition of the cell envelope undergo significant changes during the growth cycle. Furthermore, these alterations are accompanied by dramatic effects on host-parasite interactions and virulence (10–13, 18–21, 32). Similar changes, especially in the lipoidal components of the cell wall, probably occur during the growth of *Mycobacterium* (123). In general, there appear to be a correlation between the phrase of growth, the virulence, and the amount of highly unsaturated mycolic acids in the cell walls of *N. asteroides* (Beaman, unpublished data).

The mycolic acids of *Mycobacterium* are even more complex, and the envelope contains large amounts of unsaturated lipid (123). Therefore, in these organisms, the unsaturated lipids may serve as a substrate for peroxidation reactions; rendering harmless the effects of H_2O_2 (54). This concept is supported further by our observations on the chemical structure of the cell envelope of virulent and less virulent strains of *N. asteroides*. The virulent log phase cells of *N. asteroides* GUH-2 possess predominantly mycolic acids that are polyunsaturated in both the α and ß portions of the molecule. These cells are more than 1000 times more virulent for mice than cells of *N. asteroides* 10905 in the same stage of growth (19, 20). The cell walls of *N. asteroides* 10905 contain mostly saturated mycolic acids. In addition, the cells at early stages of growth of *N. asteroides* GUH-2 have more catalase (1482 units catalase per mg protein) than

cells of *N. asteroides* 10905 in the same phase of growth (200 units catalase per mg protein). Therefore, with *Mycobacterium* and *Nocardia*, the resistance of these organisms to the toxic products of oxidative metabolism of phagocytes may reside in a combined interaction among the extracellular and surface-associated SOD, large amounts of intracellular catalase and the highly unsaturated lipids in the cell envelope (10, 19, 22, 39, 40, 54, 69, 123).

The importance of superoxide dismutase and catalase in microbial resistance to the oxidative killing mechanisms of PMN leukocytes has been studied further by incubating exogenous enzyme with the organism prior to exposure to phagocytic cells. Mandell (77) studied both the virulence and catalase content of 15 strains of *Staphylococcus aureus*. It was observed that a correlation existed between the relative virulence of *S. aureus* for mice and the amount of catalase produced by that strain. Thus, those organisms that were high in catalase were virulent; whereas, the strains of *Staphylococcus* that were low in catalase were avirulent. Cells of both virulent and avirulent strains *S. aureus* were killed in vitro by human PMN leukocytes. However, high-catalase strains were more resistant to killing than low-catalase mutants (77).

Mandell found no correlation between superoxide dismutase content and either staphylococcal virulence or the relative resistance to killing by PMN neutrophils (77). When catalase was added exogenously to a low-catalase mutant of *S. aureus*, the organism was protected from the killing mechanisms of PMN leukocytes to the same degree as the more resistant high-catalase strain. Furthermore, Mandel reported that adding exogenous SOD had no effect on staphylococcal resistance to killing by human PMN phagocytes (77). In sharp contrast, Johnston et al (56) demonstrated that exogenously added superoxide dismutase protected the cells of *S. aureus, Escherichia coli,* and *Streptococcus viridans* from phagocytic killing. These investigators also noted that catalase added to the bacteria helped to protect them from being killed by human granulocytes. Complete protection of these organisms from phagocytic killing was observed only when latex particles were added in combination with the enzyme. The latex particles apparently functioned in uptake of the enzyme with the bacteria during phagocytosis (56).

There have been no previous reports of combining SOD and catalase simultaneously to determine whether these two enzymes act synergistically in protecting bacteria from oxidative killing. Therefore, we studied the relationship between catalase and SOD in nocardial resistance to the oxidative killing mechanisms of human PMN leukocytes.

As described above, the virulent *Nocardia asteroides* GUH-2 is not killed in vitro by human granulocytes, whereas more than 70% of the cells of the less virulent *N. asteroides* 10905 are killed during a 3-hour incubation with human PMN neutrophils (Beaman, unpublished data). In order to determine the role, if any, of SOD and catalase in nocardial resistance to killing by PMN phagocytes, both of these enzymes were purified from *N. asteroides* GUH-2.

The following combinations of purified nocardial SOD and catalase were then incubated for 30 minutes with single-cell suspensions of a 48-hour culture of *N. asteroides* 10905; of SOD alone, of catalase alone, and of SOD plus catalase. After 30 minutes, the organisms suspended in these enzymes prepared in Medium 199 plus 10% homologous human plasma were added to human PMN leukocytes at a ratio of one organism per phagocyte.

One set of PMN phagocytes was previously treated with chlorpromazine to inhibit the oxidative metabolic burst (greater than 99% of these cells were viable throughout the experiment as determined by trypan blue exclusion), while a second set of PMN leukocytes remained untreated. The bacterial cells were also incubated in the medium and enzyme without phagocytes. All samples were done in triplicate and all dilutions were plated in duplicate. The PMN-*Nocardia* mixtures were lysed in distilled water at 0 time, and after 90 and 180 minutes incubated at 37°C in a CO_2 incubator.

During the first 90 minutes incubation, 33.9% of the cells of *N. asteroides* 10905 were killed when incubated with PMN phagocytes alone, and 71.5% of the nocardial cells were killed after 180 minutes. Chlorpromazine-treated PMN leukocytes were unable to kill the nocardiae at 90 minutes, but they did kill 26.6% of the nocardial cells after 3 hours.

The nocardial cells pretreated with nocardial SOD were not killed in 90 minutes; however, there was a 55.1% loss in colony-forming units (CFU) after 180 minutes. The nocardial cells pretreated with nocardial catalase also had no loss of CFU at 90 minutes, and only a 36.7% decrease in CFU was observed after 3 hours. Nocardial cells that were incubated with both SOD and catalase appeared to be totally protected from oxidative killing by PMN leukocytes, because there was only a 22.5% reduction in CFU after 3 hours incubation. It should be noted that this loss in CFU was equivalent to the killing of nocardiae by chlorpromazine-treated PMN leukocytes, and it probably represented nocardial killing by nonoxidative mechanisms. Furthermore, the data suggested that the early killing (less than 90 minutes) was primarily due to oxidative metabolism, whereas the killing of cells of *Nocardia* after 3 hours was by both oxidative and nonoxidative mechanisms.

After 3 hours, 37.2% of the total killing appeared to be due to nonoxidative mechanisms. Both SOD and catalase alone were able to protect the nocardiae during initial contact with the PMN phagocytes. However, after 3 hours of incubation, SOD alone was able to provide only 36.5% protection against the oxidative metabolic burst of the PMN leukocytes. In contrast, catalase alone provided 77.5% protection against oxidative killing during prolonged incubation with PMN leukocytes. The combination of SOD and catalase were additive, and they provided complete protection against the toxic products of oxygen metabolism, because the 22.5% loss of CFU at 3 hours was the same in all groups of chlorpromazine-treated PMN leukocytes.

Exogenously added catalase was twofold more effective in protecting the

nocardial cells from oxidative killing mechanisms than SOD. These observations indicated that both the superoxide anion and hydrogen peroxide played an important role in the mechanisms whereby PMN leukocytes kill cells of *N. asteroides*.

The data presented above, utilizing the less virulent *N. asteroides* 10905, support completely the observations presented earlier concerning the role of secreted and surface-associated SOD and high concentrations of catalase in the resistance of the virulent *N. asteroides* GUH-2 to the lethal effects of PMN leukocytes (Beaman, unpublished data).

It is important to emphasize, however, that the relationship of SOD and catalase to nocardial virulence is not the sole mechanism whereby *N. asteroides* evades phagocytic killing. Indeed, there appears to be several additional nocardial components that alter macrophage, and probably PMN leukocyte, functions (11–13, 23, 31–34, 42).

Pearson et al (93) demonstrated that both promastigote and amastigote stages of the parasite *Leishmania donovani* did not interfere with the oxidative metabolic burst of human macrophages. However, they observed that the amastigote stage initiated a smaller respiratory burst than the promastigote stage (93). It has been shown that the promastigotes of both *Leishmania donovani* and *L. tropica* were susceptible to hydrogen peroxide and that most promastigotes are promptly killed by normal macrophages. The mechanisms whereby these normal macrophages killed the promastigotes of *Leishmania* were probably due to the generation of superoxide anion and hydrogen peroxide (86, 93). The amastigotes of *L. donovani* were found to contain threefold more catalase and 14-fold more glutathione peroxidase, and they were fourfold more resistant to H_2O_2 than the promastigote stage of the parasite (86).

The intracellular fate of the amastigotes corresponded with the macrophages ability to produce an oxidative metabolic burst with the generation of superoxide and hydrogen peroxide. Normal resident macrophages were not able to sufficiently respond to the amastigotes to generate large amounts of O_2^- and H_2O_2. Therefore, this stage of the parasite was not killed by these phagocytes. On the other hand, lymphokine-activated macrophages had a good respiratory burst with the generation of high levels of O_2^- and H_2O_2 in response to ingestion of the leishmanial amastigotes. These lymphokine-activated cells were very efficient at killing this stage of the parasite. However, if the oxidative activity of these macrophages was impaired by either glucose deprivation or pretreatment with phorbol myristate acetate, they lost their ability to kill the amastigotes. The addition of exogenous catalase, but not superoxide dismutase, protected the leishmanial amastigotes from being killed by the lymphokine-activated macrophages (86).

The primary differences between the amastigote and promastigote stages of *L. donovani* in their interactions with mononuclear phagocytes reside in their

relationship to the microbicidal products of oxidative metabolism, especially H_2O_2. Catalase and not superoxide dismutase appear to be involved in the pathogenesis of *L. donovani* (86).

The factors related to the tolerance of strictly anaerobic bacteria to the toxic effects of oxygen have been studied (26, 44, 47, 82, 98, 113). It was observed that the variation in susceptibility of anaerobes to the toxicity of oxygen depended mostly upon their specific content of superoxide dismutase. Thus, the anaerobes that were most susceptible to oxygen toxicity had little or no SOD, whereas those strains that were quite resistant to the lethal effects of oxygen possessed significant amounts of SOD (26, 44, 47, 98, 113). There appeared to be little correlation between oxygen toxicity in anaerobes and their content of either catalase or peroxidase.

In general, gram-negative anaerobes were more oxygen tolerant and had more SOD then gram-positive anaerobes (47, 98, 113). However, there are a few exceptions to these general observations. Certain strains of *Bacteroides fragilis* have relatively high levels of SOD and are quite resistant to the toxic effects of oxygen. It has been suggested that SOD is a virulence factor that permits the pathogenic anaerobes such as *B. fragilis* to survive in tissues until adequately reduced conditions become established that permit their growth (2, 26, 44, 47, 98, 113).

Another mechanism whereby pathogenic microorganisms may evade the killing effects of oxidative metabolism by the host's phagocytic cells could involve a failure of the organism to induce an oxidative metabolic burst during phagocytosis (67). Thus, an organism may fail either to stimulate the surface receptors that trigger oxidative metabolism or it may specifically inhibit the oxidative metabolic burst.

Kossack et al (67) found that virulent strains of *Salmonella typhi* failed to induce a significant increase in oxidative metabolism of human PMN neutrophils following phagocytosis. In contrast, avirulent strains of *S. typhi* induced a strong oxidative metabolic burst as the PMN leukocytes phagocytized the bacterial cells. The relevance of the microbicidal products of oxygen metabolism of PMN phagocytes to the virulence of *S. typhi* is questionable, however, since both avirulent strains that stimulate oxidative metabolism and virulent strains that do not are killed equally well by these granulocytes (67, 85).

Wilson et al (121) demonstrated a direct correlation between the stimulation of a respiratory burst within mononuclear phagocytes and the ability of them to kill *Toxoplasma gondii*. Thus, normal human and murine macrophages are not able to kill this parasite. Furthermore, *Toxoplasma* failed to induce an oxidative metabolic burst within these macrophages. In contrast, normal human monocytes and activated mouse peritoneal macrophages kill *Toxoplasma gondii*, and *Toxoplasma* stimulates a respiratory burst within these mononuclear phagocytes. In addition, normal human monocytes, activated mouse peritoneal

macrophages, normal human macrophages, and murine peritoneal macrophages are able to kill antibody-coated cells of *Toxoplasma,* and these antibody-coated parasites stimulate a respiratory burst within each of these phagocytes (121).

It has been demonstrated that normal PMN leukocytes and mononuclear phagocytes are unable to kill either arthroconidia or endospores of the pathogenic fungus *Coccidioides immitis* (14–17). However, either macrophages obtained from specifically immunized animals or human peripheral blood monocytes obtained from immune individuals and incubated in the presence of immune lymphocytes are able to kill both arthroconidia and endospores (14–17).

The mechanisms by which *C. immitis* resist killing by normal phagocytes, but not by activated mononuclear phagocytes, are not completely understood. There appear to be multiple factors involved. Thus, virulent cells that are not killed inhibit phagosome-lysosome fusion. Furthermore, endospores fail to induce an oxidative metabolic burst during phagocytosis by mouse peritoneal macrophages (117). Therefore, one of the mechanisms of pathogenesis of *Coccidioides immitis* may be its failure to induce a respiratory burst during phagocytosis, which results in a decrease in the microbicidal products of oxygen metabolism (117).

Proctor (95) demonstrated that preincubating human PMN leukocytes for 30 minutes with endotoxin (lipopolysaccharide: LPS) obtained from *Escherichia coli* (0111:B4) resulted in decreased superoxide formation, decreased oxygen consumption, and depression of chemiluminescence. There was a corresponding decrease in bactericidal activity of the PMN leukocytes against both *Staphylococcus aureus* and *E. coli* (95).

These effects of LPS may explain the differences observed in the lack of stimulation of the respiratory burst by virulent strains of *Shigella sonnei* phase I and *Brucella abortus* (smooth intermediate strain) as compared to the less virulent *Shigella sonnei* phase II and the less virulent *Brucella abortus* rough strain (45/20) (68, 75). The differences between both *Shigella sonnei* phase I and phase II, and *Brucella abortus* smooth intermediate (45/0) and *B. abortus* rough (45/20) reside within the chemical composition of their LPS (68, 75).

CONCLUSIONS

Both mononuclear phagocytes and granulocytes are efficient in their abilities to kill most microorganisms. They have developed both oxygen-dependent and oxygen-independent microbicidal mechanisms. The oxygen-independent mechanisms used by the phagocytes to kill microorganisms are complex, not fully understood, and beyond the scope of this review.

The oxygen-dependent microbicidal activities of "professional" phagocytes rely upon the production of reactive oxygen metabolites such as superoxide, hydroxyl radical, singlet oxygen, and hydrogen peroxide. All of these products are potentially toxic for microorganisms.

Polymorphonuclear phagocytes contain myeloperoxidase, which in the presence of hydrogen peroxide and halides such as chloride or iodide result in halogenation and killing of microorganisms. Strictly anaerobic microorganisms are killed more readily than aerobic organisms by these oxygen-dependent mechanisms because many of the oxygen-sensitive anaerobes lack superoxide dismutase, catalase, and peroxidase.

Aerobic microorganisms contain one or more of these enzymes, which function in neutralizing these toxic oxidative end products. Superoxide dismutase and catalase are generally intracytoplasmic enzymes (SOD may be associated with the periplasm in some bacteria; 122) and as a consequence they may not be readily available to protect the bacterial cell from the microbicidal effects of the superoxidative metabolic burst produced by phagocytes upon contact with the microorganism. However, it has been shown that both catalase and SOD can play a role in microbial pathogenesis, because exogenously added enzyme prevents the phagocyte-induced killing of a variety of extracellular pathogens.

Virulent strains of *Nocardia asteroides* and *Mycobacterium tuberculosis* both secrete SOD, which appears to become associated with the cell surface and produce increased intracytoplasmic levels of catalase. In contrast, the less virulent strains of *Mycobacterium* and *Nocardia* do not secrete SOD and have decreased levels of catalase. The virulence of many pathogenic microorganisms appears to be related to the relative amounts of catalase that they produce. In addition, it was observed that treatment of virulent strains of *N. asteroides* with antibody active against SOD significantly reduced their ability to survive the oxidative killing by PMN leukocytes. Also, exogenous SOD or catalase purified from virulent strains of *N. asteroides* and added to cells of the less virulent strain of *N. asteroides* greatly enhanced their resistance to the microbicidal activities of PMN phagocytes.

The results of all of these studies demonstrate that there is a relationship between the toxic oxygen metabolites produced by phagocytes and both superoxide dismutase and catalase produced by microbial cells in determining their ultimate fate in host-parasite interactions.

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Literature Cited

1. Allen, R. C., Yevich, S. J., Orth, R. W., Steele, R. H. 1974. *Biochem. Biophys. Res. Commun.* 60:909–17
2. Babior, B. M. 1978. *N. Engl. J. Med.* 298:659–68
3. Babior, B. M., Curnutte, J. T., Kepnes, R. S. 1975. *J. Lab. Clin. Med.* 85:235–44
4. Babior, B. M., Kepnes, R. S., Curnutte, J. T. 1973. *J. Clin. Invest.* 52:741–44
5. Bainton, D. F. 1973. *J. Cell Biol.* 58:249–65
6. Bainton, D. F., Ullyot, J. L., Farquhar, M. G. 1971. *J. Exp. Med.* 134:907–34
7. Baldridge, C. W., Gerald, R. W. 1933. *Am. J. Physiol.* 103:235–36
8. Baron, D. N., Ahmed, S. A. 1969. *Clin. Sci.* 37:205–8
9. Beall, G. D., Repine, J. E., Hoidal, J. R., Rasp, F. L. 1977. *Infect. Immun.* 17:117–20
10. Beaman, B. L. 1975. *J. Bacteriol.* 123:1235–53
11. Beaman, B. L. 1977. *Infect. Immun.* 15:925–37
12. Beaman, B. L. 1979. *Infect. Immun.* 26:355–61
13. Beaman, B. L. 1981. In *Actinomycetes*, ed. K. Schaal, G. Pulverer, pp. 209–20. Stuttgart: Gustav Fischer
14. Beaman, L., Benjamini, E., Pappagianis, D. 1981. *Infect. Immun.* 34:347–53
15. Beaman, L., Benjamini, E., Pappagianis, D. 1973. *Infect. Immun.* 39:1201–7
16. Beaman, L., Holmberg, C. 1980. *Infect Immun.* 28:594–600
17. Beaman, L., Holmberg, C. 1980. *Infect. Immun.* 29:1200–1
18. Beaman, B. L., Bourgeois, A. L. 1981. *J. Clin. Microbiol.* 14:574–78
19. Beaman, B. L., Bourgeois, A. L., Moring, S. E. 1981. *J. Bacteriol.* 148:600–9
20. Beaman, B. L., Maslan, S. 1978. *Infect. Immun.* 20:290–95
21. Beaman, B. L., Maslan, S., Scates, S., Rosen, J. 1980. *Infect. Immun.* 28:185–89
22. Beaman, B. L., Scates, S. M., Moring, S. E., Deem, R., Misra, H. P. 1983. *J. Biol. Chem.* 258:91–96
23. Black, C., Beaman, B. L., Donovan, R. M., Goldstein, E. 1983. *J. Infect. Dis.* 148:117–24
24. Bullen, J. J., Armstrong, J. A. 1979. *Immunol.* 36:781–91
25. Bullen, J. J., Rogers, H. J., Leigh, L. 1972. *Br. Med. J.* 1:69–75
26. Carlson, J., Wrethen, J., Beckman, G. 1977. *J. Clin. Microbiol.* 6:280–84
27. Cohen, M. S., Isturiz, R. E., Malechn, H. L., et al. 1981. *Am. J. Med.* 71:59–66
28. Cohn, Z. A. 1978. *J. Immunol.* 121:813–16
29. Cohn, Z. A., Wiener, E. 1963. *J. Exp. Med.* 118:991–1008
30. Curnutte, J. T., Whitten, D. M., Babior, D. M. 1974. *N. Engl. J. Med.* 290:593–97
31. Davis-Scibienski, C., Beaman, B. L. 1980. *Infect. Immun.* 28:610–19
32. Davis-Scibienski, C., Beaman, B. L. 1980. *Infect. Immun.* 29:24–29
33. Davis-Scibienski, C., Beaman, B. L. 1980. *Infect. Immun.* 30:578–87
34. Deem, R., Doughty, F. A., Beaman, B. L. 1983. *J. Immunol.* 130:2401–6
35. Drath, D. B., Karnovsky, M. L. 1975. *J. Exp. Med.* 141:257–62
36. Drazin, R. E., Lehrer, R. I. 1977. *Infect. Immun.* 17:382–88
37. Efrati, C., Sacks, T., Ne'eman, N., Lahov, M., Ginsburg, I. 1976. *Inflammation* 1:371–407
38. Elsbach, P., Weiss, J. 1983. *Rev. Infect. Dis.* 5:843–53
39. Filice, G. A. 1983. *J. Infect. Dis.* 148:861–67
40. Filice, G. A., Beaman, B. L., Krick, J. A., Remington, J. S. 1980. *J. Infect. Dis.* 142:432–38
41. Filice, G. A., Beaman, B. L., Krick, J. A., Remington, J. S. 1981. In *Heterogeneity of Mononuclear Phagocytes*, ed. O. Forester, M. Landy, pp. 455–62 London: Academic
42. Filice, G. A., Beaman, B. L., Remington, J. S. 1980. *Infect. Immun.* 27:643–49
43. Goldstein, I. M., Cerquiera, M., Lund, S., et al. 1977. *J. Clin. Invest.* 59:249–54
44. Gregory, E. M., Moore, W. E., Holdeman, L. V. 1978. *Appl. Environ. Microbiol.* 35:988–91
45. Harrison, J. E., Schultz, J. 1976. *J. Biol. Chem.* 251:1371–74
46. Heise, E. R., Myrick, Q. N. 1967. *RES* 4:510–23
47. Hewitt, J., Morris, J. G. 1975. *FEBS. Lett.* 15:315–18
48. Hirsch, J. G. 1956. *J. Exp. Med.* 103:589–611
49. Hirsch, J. G. 1960. *J. Exp. Med.* 111:323–37
50. Hocking, W. G., Golde, D. W. 1979. *N. Eng. J. Med.* 301:580–87
51. Holmes, B., Good, R. B. 1972. *RES* 12:216–37
52. Holmes, B., Page, A. R., Good, R. A. 1967. *J. Clin. Invest.* 46:1422–32

53. Iyer, G. Y. N., Islam, M. F., Quaslel, J. H. 1961. *Nature* 192:535–541
54. Jackett, P. S., Aber, V. R., Lowrie, O. B. 1978. *J. Gen. Microbiol.* 104:37–45
55. Johnston, R. B., Baehner, R. L. 1971. *Pediatrics* 48:730–39
56. Johnston, R. B., Godzik, G. A., Cohn, Z. A. 1978. *J. Exp. Med.* 148:115–27
57. Johnston, R. B., Keele, B. B., Misra, H. P., Lehmeyer, J., et al. 1975. *J. Clin. Invest.* 55:1357–72
58. Johnston, R. B., Lehmeyer, J. E., Guthrie, L. A. 1976. *J. Exp. Med.* 143:1551–56
59. Karnovsky, M. L., Lazdins, J. K. 1978. *J. Immunol.* 121:809–13
60. Kirkpatrick, C. H., Green, I., Rich, R. R., Schade, A. L. 1971. *J. Infect. Dis.* 124:539–44
61. Klebanoff, S. J. 1967. *J. Exp. Med.* 126:1063–78
62. Klebanoff, S. J. 1968. *J. Bacteriol.* 95:2131–38
63. Klebanoff, S. J. 1975. *Semin. Hematol.* 12:117–42
64. Klebanoff, S. J. 1980. *Ann. Int. Med.* 93:480–89
65. Klebanoff, S. J., Hamon, C. B. 1972. *RES* 12:170–96
66. Klebanoff, S. J., Pincus, S. H. 1971. *J. Clin. Invest.* 50:2226–29
67. Kossack, R. E., Guerrant, R. L., Densen, P., Schaedelin, J., Mandell, G. L. 1981. *Infect. Immun.* 31:674–78
68. Kreutzer, D. L., Dreyfus, L. A., Robertson, D. C. 1979. *Infect. Immun.* 23:737–42
69. Kusunose, E., Ichihara, K., Noda, Y., Kusunose, M. 1976. *J. Biochem.* 80:1343–52
70. Lehrer, R. I. 1972. *J. Clin. Invest.* 51:2566–72
71. Lehrer, R. I. 1975. *J. Clin. Invest.* 55:338–46
72. Lehrer, R. I., Cline, M. J. 1969. *J. Clin. Invest.* 48:1478–88
73. Lehrer, R. I., Ferrari, L. G., Patterson-Delafield, J. 1977. *Clin. Res.* 25:490A
74. Lehrer, R. I., Ladra, K. M., Hake, R. B. 1975. *Infect. Immun.* 11:1226–34
75. Madonna, G. S., Allen, R. C. 1981. *Infect. Immun.* 32:153–59
76. Mandell, G. L. 1974. *Infect. Immun.* 9:337–41
77. Mandell, G. L. 1975. *J. Clin. Invest.* 55:561–66
78. Mandell, G. L., Hook, E. W. 1969. *J. Bacteriol.* 100:531–32
79. Masson, P. L., Heremans, J. F., Prignot, J. J., Wauters, G. 1966. *Thorax* 21:538–44
80. Matheson, I. B. C., Etheridge, R. D., Kratowich, N. R., Lee, J. 1975. *Photochem. Photobiol.* 21:165–71
81. McCall, C. E., DeChatelef, L. R., Cooper, M. R., Ashburn, P. 1971. *J. Infect. Dis.* 124:194–98
82. McCord, J. M., Keele, B. B., Fredovich, I. 1971. *Proc. Natl. Acad. Sci. USA* 68:1024–27
83. McRipley, R. J., Sbarra, A. J. 1967. *J. Bacteriol.* 94:1417–24
84. McRipley, R. J., Sbarra, A. J. 1967. *J. Bacteriol.* 94:1425–30
85. Miller, R. M., Barbus, J., Hoenik, R. G. 1972. *Science* 175:1010–11
86. Murray, H. W. 1982. *J. Immunol.* 129:351–56
87. Murray, H. W., Cohn, Z. A. 1980. *J. Exp. Med.* 152:1596–1609
88. Nathan, C. F., Nogueira, N., Juangbhanich, C., Ellis, J. Cohn, Z. 1979. *J. Exp. Med.* 149:1056–68
89. Nathan, C. F., Root, R. K. 1977. *J. Exp. Med.* 146:1648–62
90. Nelson, R. D., Mills, E. L., Simmons, R. L., Quie, P. G. 1976. *Infect. Immun.* 14:129–34
91. Oram, J. D., Reiter, B. 1968. *Biochim. Biophys. Acta* 170:351–65
92. Oren, R., Farnham, A. E., Saito, K., Milofsky, E., Karnovsky, M. L. 1963. *J. Cell Biol.* 17:487–501
93. Pearson, R. D., Harcus, J. L., Symes, P. H., Romito, R., Donowitz, G. P. 1983. *J. Immunol.* 129:1282–87
94. Pitt, J., Bernheimer, H. P. 1974. *Infect. Immun.* 9:48–52
95. Proctor, R. A. 1979. *Infect. Immun.* 25:912–21
96. Quie, P. G., White, J. G., Holmes, B., et al. 1967. *J. Clin. Invest.* 46:668–79
97. Rodey, G. E., Park, B. H., Windhorst, D. B., et al. 1969. *Blood* 33:813–20
98. Rolfe, R. D., Hentges, D. J., Campbell, B. J., Barrett, J. T. 1978. *Appl. Environ. Microbiol.* 36:306–13
99. Root, R. K., Cohen, M. S. 1981. *Rev. Infect. Dis.* 3:565–98
100. Rosen, H., Klebanoff, S. J. 1976. *J. Clin. Invest.* 58:50–60
101. Rosen, H., Klebanoff, S. J. 1977. *J. Biol. Chem.* 252:4803–10
102. Rosen, H., Klebanoff, S. J. 1979. *J. Clin. Invest.* 64:1725–29
103. Sagone, A. L., King, G. W., Metz, E. N. 1976. *J. Clin. Invest.* 57:1352–58
104. Sasada, M., Johnston, R. B. Jr. 1980. *J. Exp. Med.* 152:85–98
105. Sbarra, A. J., Karnovsky, M. L. 1959. *J. Biol. Chem.* 234:1355–62
106. Segal, A. W. 1974. *Lancet* 2:1248–52
107. Selvaraj, R. J., Paul, B. B., Strauss, R. R., Jacobs, A. A., Sbarra, A. J. 1974. *Infect. Immun.* 23:522–31

108. Shohet, S. B., Pitt, J., Baehner, R. L., Poplack, D. G. 1974. *Infect. Immun.* 10:1321–28
109. Spitznagel, J. K. 1977. *The Granulocyte: Function and Clinical Utilization*, pp. 103–31. N.Y.: Liss. 341 pp.
110. Spitznagel, J. K., Okamura, N. 1983. *Host-Defenses to Intracellular Pathogens*, pp. 5–17. N.Y.: Plenum. 538 pp.
111. Stahelin, H., Suter, E., Karnovsky, M. L. 1956. *J. Exp. Med.* 104:121–36
112. Strauss, R. R., Paul, B. B., Jacobs, A. A., Sbarra, A. J. 1971. *Infect. Immun.* 3:595–602
113. Tally, F. P., Goldin, B. R., Jacobus, N. V., Gorbach, S. L. 1977. *Infect. Immun.* 16:20–25
114. Tauber, A. I., Babior, B. M. 1978. *Photochem. Photobiol.* 28:701–9
115. Thomas, E. L. 1979. *Infect. Immun.* 23:522–31
116. Thomas, E. L., Aune, T. M. 1978. *Agents Chemother.* 13:1006–10
117. Vollger, H. F., Langton, B. C., Beaman, L., Pappagianis, P., Benjamini, E. 1982. *Fed. Proc.* 41:963
118. Wang-Iverson, P., Prywzansky, K. B., Spitznagel, J. K., Cooney, M. H. 1978. *Infect. Immun.* 22:945–55
119. Welch, D. F., Sword, C. P., Brehm, S., Duxanis, D. 1979. *Infect. Immun.* 23:863–72
120. Weser, U., Paschen, W., Younes, M. 1975. *Biochem. Biophys. Res. Commun.* 66:769–77
121. Wilson, C. B., Tsai, V., Remington, J. S. 1980. *J. Exp. Med.* 151:328–46
122. Yost, F. J. Jr., Fredoerch, I. 1974. *Arch. Biochem. Biophys.* 161:395–401
123. Youmans, G. P. 1979. *Tuberculosis.* Philadelphia: Saunders 511 pp.
124. Zeya, H. I., Spitznagel, J. L. 1966. *Science* 154:1049–51

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METABOLISM OF METHYLGLYOXAL IN MICROORGANISMS

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INTRODUCTION

Methylglyoxal (2-oxopropanal) has had a checkered history in biology. About 50 years ago it was widely believed to be a key intermediate in the pathway for glucose breakdown in animals, plants, and microorganisms because it was

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readily produced from hexosediphosphate by cell-free extracts (26, 44, 56, 57, 62, 75, 80, 88). But as the 1930s progressed the role of phosphorylated glyceric acids in glucose breakdown became apparent (21, 49, 53, 86) and by the late 1940s the formation of methylglyoxal had been dismissed as an artifact (48).

Meyerhof & Lohmann (49) showed that the triosephosphates were converted to methylglyoxal in nonenzymatic acid–catalyzed reactions, and because the incubation mixtures in which methylglyoxal had earlier been identified were invariably treated with trichloroacetic acid, the appearance of methylglyoxal was considered to be a consequence of the acid-catalyzed breakdown of accumulated triosephosphates. This view was widely held in the 1950s, and the chemical production of methylglyoxal from triosephosphates was utilized in a colorimetric assay for fructose bisphosphate aldolase activity (46, 72).

In 1952 Entner & Doudoroff detected methylglyoxal accumulation during glucose catabolism by iodoacetate-poisoned cells of *Pseudomonas saccharophila* (22). The methylglyoxal appeared to be produced from glyceraldehyde 3-phosphate under these conditions, and one mole of glucose was converted to one mole of pyruvate and one mole of methylglyoxal. They regarded this as a new phenomenon, which revived the question of the biological role of methylglyoxal in glucose catabolism. However, no enzymatic reaction producing methylglyoxal from glycolytic intermediates was identified during this period, and interest in methylglyoxal as an intermediate of glucose catabolism went into rapid decline.

Methylglyoxal production during glucose catabolism by an *Escherichia coli* strain was reported in 1964, and its enzymatic formation from glyceraldehyde 3-phosphate was also described (85). No further reports appeared until 1970, but developments since then have resulted in a revival of interest in the possible glycolytic role of methylglyoxal (12).

This renewed interest stemmed from investigations of gluconeogenesis from glycerol in *E. coli* mutants lacking triosephosphate isomerase (EC 5.3.1.1). Such strains could produce dihydroxyacetone phosphate from glycerol but could not then isomerise it to give glyceraldehyde 3-phosphate. Cell-free extracts from the mutant formed methylglyoxal from dihydroxyacetone phosphate but not from glyceraldehyde 3-phosphate (14, 36). It was quickly established that the enzyme in question, methylglyoxal synthase (EC 4.2.99.11), was present in a variety of bacteria and that its activity was extremely sensitive to inhibition by orthophosphate (35). These observations were soon confirmed independently (25).

There have also been reports of the formation of methylglyoxal during the catabolism of certain amino acids (20, 28) and other compounds (31, 79), so the production of methylglyoxal now appears to be firmly established as a bona fide property of some microorganisms, at least.

It is therefore appropriate to consider this "born again" metabolite 50 years on from its heyday in an attempt to assess its significance as an intermediate of microbial metabolism.

A METHYLGLYOXAL BYPASS OF THE EMBDEN-MEYERHOF PATHWAY

In the 1920s and 1930s one of the main arguments in support of a glycolytic role for methylglyoxal was the widespread occurrence, at high activity, of an enzyme system that converted methylglyoxal into lactate (16, 54). This system is now known (64) to be composed of two enzymes—glyoxalase I (EC 4.4.1.5: *S*-lactoylglutathione methylglyoxal-lyase) and glyoxalase II (EC 3.1.2.6; hydroxyacylglutathione hydrolase)—whose sequential action produces D-lactate, in contrast to the L-lactate formed by many glycolysing systems.

Several bacteria, including *E. coli*, were known to possess glyoxalase activity (55, 73, 87). They also possessed flavin-linked D- and L-lactate dehydrogenases (EC 1.1.2.4; 1.1.2.3) that form pyruvate from lactate under aerobic conditions (40). It is therefore possible, once methylglyoxal has been produced, to transform it into pyruvate via lactate, thereby effecting a bypass of the Embden-Meyerhof sequence for the formation of pyruvate from phosphorylated three-carbon intermediates (Figure 1). The conversion of glycerol to pyruvate, observed during studies with triosephosphate isomerase–negative mutants of *E. coli*, involves the operation of this sequence (14).

Although methylglyoxal synthase has been detected in a number of bacteria (5, 13, 35, 81), the further catabolism of methylglyoxal in these organisms has often not been investigated. *E. coli* has received the most attention, and even here the extent to which the sequence is used during normal catabolism is difficult to evaluate. The enzymes are all formed constitutively (14), but it is apparent from studies with mutants blocked in various reactions of the Embden-Meyerhof sequence that when triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3), or enolase (EC 4.2.1.11) is defective, no persistent growth on glucose occurs (2, 33, 37). This indicates that the methylglyoxal bypass is not normally an alternative to the corresponding Embden-Meyerhof reactions, although it is always possible that in such mutants the defects in the Embden-Meyerhof pathway lead to the accumulation of phosphorylated glycolytic intermediates, and such compounds are known to be inhibitory to growth (7, 15, 23).

One kind of glycolytic mutant, that lacking pyruvate kinase activity *(pykAF)*, grows apparently normally on exogenous glucose, but it is likely that the phosphoenolpyruvate-dependent phosphotransferase system (43) is instrumental in permitting such growth (27). However, these *pykAF* mutants grow very slowly when glucose is produced internally from catabolism of

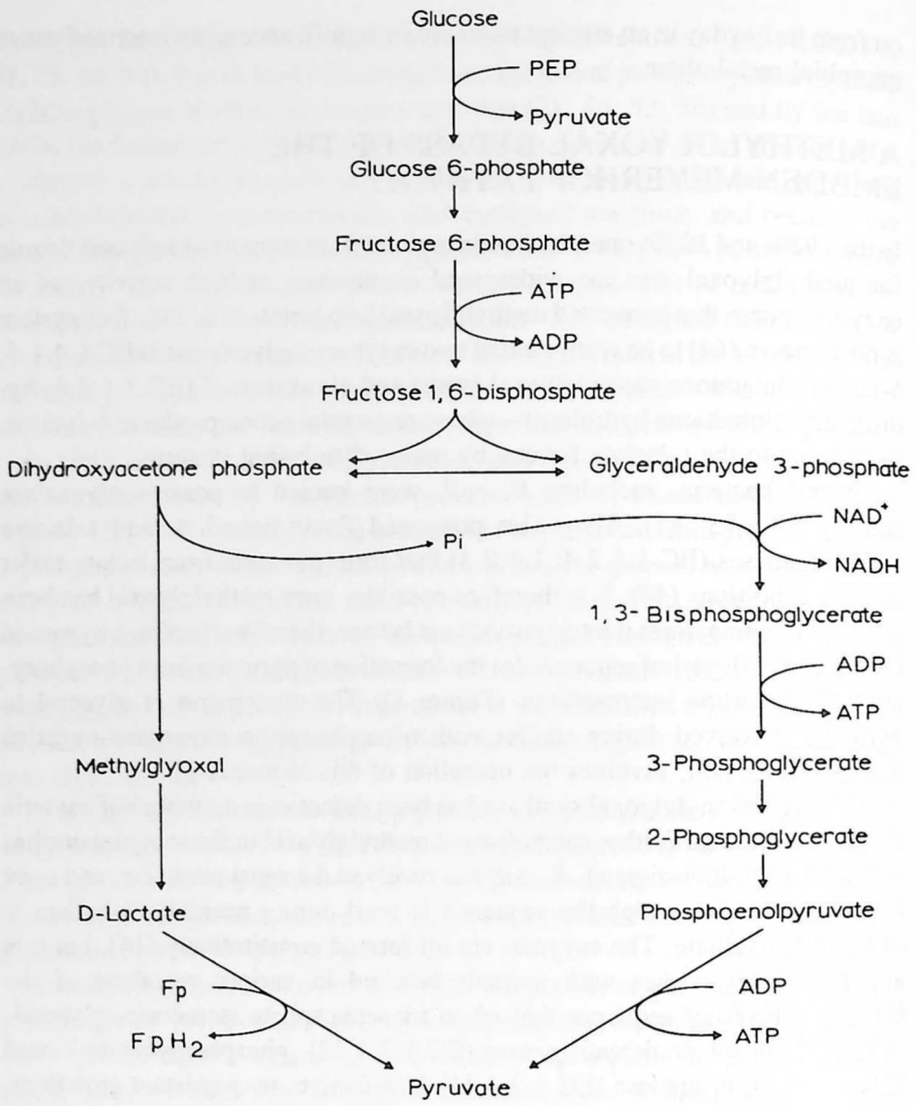


Figure 1 Representation of the Embden-Meyerhof pathway with the methylglyoxal bypass.

maltose or lactose, and under these conditions methylglyoxal is produced (27). Whether the methylglyoxal bypass is responsible for this slow growth is not known, but it obviously is a possibility.

In addition to this situation for *pykAF* mutants, methylglyoxal can be detected in the growth medium in other circumstances. Triosephosphate isomerase–negative mutants growing on gluconate, fructose bisphosphate aldolase–negative mutants growing on glycerol, and wild-type cells growing on glucose 6-phosphate show methylglyoxal at 0.1–0.2 mM concentration in the growth medium (12).

Assessment of the operation of the methylglyoxal bypass in normal cells is not straightforward. Catabolism of [1-^{14}C]glucose in the triosephosphate isomerase mutant of *E. coli* yields [1-^{14}C]dihydroxyacetone phosphate. This, in turn, leads to the production of methyl-labeled methylglyoxal and, through the glyoxalase system, to methyl-labeled lactate. Since the lactate produced from [1-^{14}C]glucose by the Embden-Meyerhof sequence is also methyl-labeled, no information on the proportion of lactate produced by the bypass sequence can be obtained from experiments with [1-^{14}C]glucose.

However, when an *E. coli* mutant defective in D-lactate dehydrogenase was grown on 5 mM glucose 6-phosphate, about 1 mM D-lactate was present in the medium at the end of growth (12). Some of this lactate may have been formed by reduction of pyruvate, but no lactate was found after growth of the mutant on glucose, thereby supporting the view that the lactate accumulated during growth on glucose 6-phosphate was produced via the methylglyoxal bypass.

Further evidence for the operation of the sequence has come from studies of pyridoxol formation (84). From experiments utilizing [^{14}C]glycerol and [^{14}C]glucose it was concluded that the C-2',-2 carbon atoms of the pyridoxol molecule were derived from pyruvate that had not been produced from glyceraldehyde 3-phosphate by the Embden-Meyerhof reactions. Methylglyoxal was readily incorporated into the C-2',-2 atoms of pyridoxol, and it was concluded that under the conditions of the experiments dihydroxyacetone phosphate was converted to pyruvate by the bypass sequence.

Although most of the recent work on the production and utilization of methylglyoxal has concerned *E. coli*, a glycolytic bypass involving methylglyoxal has been proposed for various yeasts (4). A number of yeasts were found to have methylglyoxal synthase at an activity equivalent to that of *E. coli* and were capable of forming lactate by the glyoxalase system. Because D-lactate dehydrogenase activity was observed in only certain yeasts, the general operation of the methylglyoxal bypass in yeast remains to be established.

This situation has been complicated by a recent report that, under certain conditions, *Saccharomyces cerevisiae* did indeed produce methylglyoxal, but no methylglyoxal synthase activity could be detected (61). The authors concluded that the methylglyoxal was produced nonenzymatically from glyceraldehyde 3-phosphate.

Several years ago the production of methylglyoxal from glyceraldehyde by a *Pseudomonas* sp. was reported (68). This reaction was proposed as a step in a sequence of reactions for the conversion of glyceraldehyde 3-phosphate to pyruvate during growth on gluconate and, as already described, *P. saccharophila* was known to accumulate methylglyoxal when poisoned with iodoacetate. This reaction has not been described further, and because methylglyoxal synthase has also been reported to be present in *P. saccharophila* (11) and

in *P. doudoroffi* (5), the significance of this formation of methylglyoxal from glyceraldehyde remains to be established.

CYTOTOXIC NATURE OF METHYLGLYOXAL

A significant feature of methylglyoxal is its cytotoxic effect (19, 25, 91). The first reports of methylglyoxal cytotoxicity indicated that the primary site of attack was on protein synthesis (18). However, another report suggested that protein synthesis, DNA synthesis, and RNA synthesis were all strongly inhibited (42). More recently, from experiments utilizing synchronous cultures of *E. coli*, it has been proposed that in addition to protein synthesis methylglyoxal also inhibits the initiation of DNA synthesis but not DNA chain elongation. It was concluded that the primary site of action of methylglyoxal was on protein synthesis (24).

It has been suggested that methylglyoxal inhibits protein synthesis through interaction with the ribosome (58). In this situation it may be pertinent to note that methylglyoxal reacts rapidly with 7-methylguanosine (41) and that there is a 7-methylguanine residue in 16S and in 23S RNA (9, 10).

The extent of inhibition by methylglyoxal depends on the cell density at the time of addition and upon the concentration used. A methylglyoxal concentration of 0.25 mM completely inhibited the growth of an *E. coli* culture containing 3×10^6 cells ml^{-1}, but concentrations around 1 mM were needed to inhibit the growth of a culture containing 3×10^8 cells ml^{-1} (24).

This variation in the amount of methylglyoxal needed for inhibition is due to an inherent ability of the cells to detoxify methylglyoxal through the activity of the glyoxalase system. Nevertheless this system can be swamped. Any cell that produces methylglyoxal lives dangerously and is likely to have developed mechanisms for the careful regulation of its methylglyoxal concentration.

ENZYMATIC FORMATION OF METHYLGLYOXAL

Studies on Methylglyoxal Synthase

REACTION MECHANISM Methylglyoxal synthase catalyzes an apparently irreversible reaction (35) in which dihydroxyacetone phosphate is converted into methylglyoxal and inorganic orthophosphate (Pi):

$$\text{Dihydroxyacetone phosphate} \rightarrow \text{Methylglyoxal} + \text{Pi}$$

The mechanism of methylglyoxal formation has been studied both for the isolated enzyme (38, 74, 90) and for whole cells (Emerson & Cooper, unpublished observations). All the results suggest that the hydroxymethyl group of dihydroxyacetone phosphate is oxidized to give the aldehyde group, while dephosphorylation and reduction at carbon one leads to the methyl group.

Proton abstraction at carbon three of dihydroxyacetone phosphate is stereospecific for the *pro-S* hydrogen of the hydroxymethyl group, with no "intramolecular" transfer of the *pro-R* hydrogen to give the methyl group of methylglyoxal. The proton addition to form the methyl group was nonstereospecific, so it was proposed that the enolaldehyde (which ketonizes in solution) rather than the enediolphosphate is the true enzyme product (74).

Recently it has been shown that methylglyoxal synthase, unlike triosephosphate isomerase and fructose bisphosphate aldolase (EC 4.1.2.13), accepts the *trans*-enediolphosphate rather than the *cis*-enediolphosphate as a substrate (38). Thus the enzyme catalyzes a stereospecific abstraction of a proton followed by a β-elimination of orthophosphate from the resulting enediolphosphate.

Haloacetol phosphates have been used as reactive derivatives of dihydroxyacetone phosphate to try to identify the active site of methylglyoxal synthase (89). 3-Bromo- and 3-iodoacetol phosphate irreversibly inactivated the enzyme, but 3-chloroacetol phosphate acted only as a competitive inhibitor.

Surprisingly, neither dihydroxyacetone phosphate nor Pi fully protected the enzyme against inactivation. The enzyme totally inactivated by 3-iodoacetol phosphate apparently bound four equivalents per mole of enzyme. This was interpreted as indicating some nonselective modification, or selective modification of nonessential residues, in addition to modification of the active site (89). However, no specific active-site residue has yet been firmly identified.

INHIBITION OF ACTIVITY When the time-course for methylglyoxal formation was measured, the initial linear rate of formation declined rapidly. When either methylglyoxal or Pi at 1 mM concentration was included to see if the inhibition was caused by accumulated end products, methylglyoxal caused no inhibition but Pi had a potent effect.

Recently it was shown that methylglyoxal at 100 mM concentration had no inhibitory effect (38). When other phosphate-containing metabolites were tested at 1 mM concentration, α-glycerophosphate, fructose 1,6-bisphosphate, fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate, 6-phosphogluconate, ribose 5-phosphate, ATP, AMP, NAD^+, and NADH all had little or no effect, causing from 0–10% inhibition (35).

However, 3-phosphoglycerate and phosphoenolpyruvate were more effective, giving 50% and 70% inhibition, respectively. Inorganic pyrophosphate (PPi) was almost as effective as Pi, causing 95% inhibition. This inhibition was not due to formation of Pi from PPi.

The mode of action of these inhibitors became apparent from kinetic studies of methylglyoxal synthase. When activity was measured at various concentrations of dihydroxyacetone phosphate, the enzyme showed normal Michaelis-Menten kinetics with a K_m ranging from 0.09 mM to 0.47 mM (11, 36, 81).

However, the presence of Pi at low concentrations (0.2–0.5 mM) caused the

response to dihydroxyacetone phosphate to become sigmoidal, and raising the Pi concentration led to an increasingly pronounced sigmoidal response (36). Arsenate, an analogue of Pi, caused a similar change in the response to dihydroxyacetone phosphate, but at a given concentration was less effective than Pi. The inhibitory effect of Pi was also observed when the enzyme was assayed in vivo in toluene-treated cells, suggesting that the response was not an artifact caused by purification of the enzyme (Thomas & Cooper, unpublished observations).

Inhibition by PPi was competitive with respect to dihydroxyacetone phosphate, and K_i values of 0.048 mM, 0.083 mM, and 0.095 mM have been reported for the enzyme from three different organisms (11, 36, 81). For the enzyme from *P. saccharophila* (11) and *Proteus vulgaris,* (81) inhibition by 3-phosphoglycerate and phosphoenolpyruvate was also competitive, with K_i values of 96 μM and 29 μM, respectively, for the *P. saccharophila* enzyme. Inhibition of the *E. coli* enzyme by 3-phosphoglycerate and phosphoenolpyruvate did not follow simple competitive kinetics (36).

It is noteworthy that glyceraldehyde 3-phosphate was reported to be a competitive inhibitor of the *P. vulgaris* enzyme, even though it was not a substrate for methylglyoxal formation (81). It was not inhibitory for the *E. coli* enzyme (36).

OTHER PROPERTIES Methylglyoxal synthase has been detected in crude extracts of all the Enterobacteriaceae and Enterobacteriaceae-like organisms that have been tested (13). It has also been found in two aerobes, *P. saccharophila* (11) and *P. doudoroffi* (5), and in two anaerobes, *Clostridium tetanomorphum* and *Clostridium pasteurianum* (13).

Interestingly, it has been claimed that the enzyme was not present in *E. coli, P. vulgaris,* and *Enterobacter aerogenes* after anaerobic growth (81). It has been reported to be present in a number of yeasts (4) but could not be detected in a strain of *S. cerevisiae* that produced methylglyoxal during glycerol catabolism (61).

In the great majority of cases the enzyme has simply been sought in crude extracts, but it has been purified extensively from *E. coli* K12 (36) and crystallised from *P. vulgaris* (81). In both cases standard purification procedures were employed but the *E. coli* enzyme was found to be rapidly inactivated during purification unless Pi (1 mM) was included in the buffers used.

No information on the stability of the *P. vulgaris* enzyme in the absence of Pi was presented, but 1 mM Pi was included in the buffers along with EDTA and 2-mercaptoethanol. Pi at 1 mM concentration was shown to affect the electrophoretic mobility of the purified *E. coli* enzyme, suggesting that it caused a change in conformation (36). However, no binding studies have appeared and the mechanism of interaction between Pi and methylglyoxal synthase remains unknown.

As already indicated, the enzymes from *E. coli* and *P. vulgaris* showed many properties in common, and both were optimally active around pH 7.5–7.7. There were, however, some differences. The mol wt of the *P. vulgaris* enzyme was 135,000, and it was composed of two apparently identical monomers, mol wt 66,000 ± 3,000. The enzyme from *E. coli* (and *P. saccharophila*) was found by gel filtration to have a mol wt of 67,000, but no information was presented on whether this was a monomer or dimer. One curious aspect was the difference in specific activity between the *E. coli* enzyme, estimated to be about 70% pure (maximum specific activity around 500), and the crystalline *P. vulgaris* enzyme, with a maximum specific activity of 9.

Glyceraldehyde 3-Phosphate and Glyceraldehyde as Possible Precursors of Methylglyoxal

Reports on an enzyme-catalyzed formation of methylglyoxal from glyceraldehyde 3-phosphate and from glyceraldehyde have appeared (68, 85). Since these reports have not subsequently been extended, it is useful to consider possible alternative explanations of the findings.

In the case of methylglyoxal production from glyceraldehyde 3-phosphate by extracts of *E. coli* strain RC-1 (85), the most likely explanation is that triosephosphate isomerase was present in the crude extracts used, thereby producing dihydroxyacetone phosphate, the substrate for methylglyoxal synthase. It was noted previously that methylglyoxal could be formed from glyceraldehyde 3-phosphate by crude extracts of *E. coli,* but, significantly, there was no methylglyoxal formed from glyceraldehyde 3-phosphate by a crude extract from a triosephosphate isomerase–negative mutant (14).

An alternative proposal for the formation of methylglyoxal from glyceraldehyde by a glyceraldehyde dehydratase is more difficult to identify. The nonenzymatic formation of methylglyoxal from glyceraldehyde has been well documented and has been shown to occur under certain physiological conditions (67).

It has been reported that extracts from *P. saccharophila* form methylglyoxal from dihydroxyacetone phosphate but not from glyceraldehyde (11), and the organism reported to have glyceraldehyde dehydratase appears also to have methylglyoxal synthase activity (12). Thus, the significance of methylglyoxal production from glyceraldehyde remains to be established.

Acetol Dehydrogenase

Methylglyoxal can be produced by reactions quite separate from those of glucose degradation. An enzyme forming methylglyoxal from acetol (1-hydroxyacetone), an intermediate in the catabolism of isopropanol and acetone, has been described recently (79). The enzyme, acetol dehydrogenase (EC 1.1.1. −), uses NAD^+ as the cosubstrate. Its activity in acetone-grown cells was more than 20-fold greater than that in cells grown on acetate or succinate.

The enzyme in crude extracts was completely inactivated within 24 hr when kept at 3°C, but incorporation of 10% (v/v) ethanol into the extract stabilized the activity such that no loss was observed during 70 hr at 3°C. However, even the presence of ethanol did not permit the recovery of significant activity after DEAE-cellulose chromatography; clearly, the enzyme is labile.

Methylglyoxal from Aminoacetone

The production of methylglyoxal from aminoacetone through the action of a transaminase or oxidase in *Staphylococcus aureus* was proposed as part of a cycle for the oxidation of glycine or threonine (20). However, no such enzyme activity from *S. aureus* has yet been described. Similarly, the production of methylglyoxal during the catabolism of aminoacetone by a pseudomonad has been proposed (32). Methylglyoxal aminotransferase could not be detected, but some aminoacetone oxidase activity was reported (31). However, there has been no further description of this enzyme.

REACTIONS FOR THE UTILIZATION OF METHYLGLYOXAL

Methylglyoxal can be transformed to D-lactate or to pyruvate. In both instances the likely initial substrate is the hydrated form of methylglyoxal:

$$CH_3\text{-}CO\text{-}CH(OH)_2$$

because α-ketoaldehydes are known to exist predominantly in the hydrated form. The formation of D-lactate results from the action of the glyoxalase system, while pyruvate is produced by methylglyoxal dehydrogenase (EC 1.1.1. −) or possibly by formaldehyde dehydrogenase (EC 1.2.1.1).

The Glyoxalase System

Although the glyoxalase system was discovered more than 70 years ago (16, 54) and appears to be very widely distributed (34), it has not been studied extensively, perhaps because its true physiological role was uncertain. After the realization that the glyoxalase system was two separate enzymes, glyoxalase I and glyoxalase II, most investigations have concentrated on glyoxalase I; little attention has been paid to glyoxalase II. The consecutive operation of glyoxalase I and glyoxylase II converts methylglyoxal to D-lactate and involves glutathione as a coenzyme.

The reaction mechanism is unusual in that methylglyoxal and glutathione first combine in a nonenzymatic reaction to form an adduct, almost certainly a hemimercaptal, which is the true substrate for glyoxalase I (83). Glyoxalase

I from yeast has been crystallized (65). It is a small protein, mol wt 48,000, and appears to have a fairly broad substrate specificity towards α-ketoaldehydes (83). The K_m for the methylglyoxal-glutathione adduct is 3×10^{-4} M (83).

Some recent studies have suggested that the glyoxalase system does play a physiological role in methylglyoxal catabolism. A number of reports show that when *E. coli* is exposed to inhibitory concentrations (1 mM) of methylglyoxal, mutants are selected in which the exogenous methylglyoxal is utilized more rapidly (12, 25, 51). These mutants show six to ten times higher activity of glyoxalase I with little or no increase in glyoxalase II (12). This implies that the two glyoxalase enzymes are not in a single transcriptional unit.

Although such methylglyoxal-resistant mutants can be isolated readily, the phenotype is complex because in our hands they grow little better, initially, than the parent strains when they are transferred back to fresh media containing 1 mM methylglyoxal. In many ways they behave like some antibiotic-resistant mutants in that they need to reduce the concentration of inhibitor before they grow readily. It seems quite likely that the increased activity of glyoxalase I will remove methylglyoxal by transforming it to lactoylglutathione, which may then simply be excreted because *E. coli* cells are known to make relatively large amounts of glutathione (3). In fact, a procedure for the preparation of glutathione by immobilized cells of a methylglyoxal-resistant mutant has been described (77).

More support for the physiological operation of the glyoxalase system was obtained when whole cells of wild-type *E. coli* were incubated with 5 mM methylglyoxal. There was virtually quantitative conversion of the methylglyoxal to give D-lactate, and it seemed from this that the glyoxalase system alone was responsible for methylglyoxal catabolism in *E. coli* (14).

However, when cell-free extracts were used, the rate of methylglyoxal utilization was 75% lower than that of whole cells, and pyruvate was produced rather than D-lactate. Such cell-free extracts readily formed pyruvate from D-lactate, so it was concluded that the pyruvate arose from the oxidation of lactate (14). For *E. coli,* glyoxalase I and glyoxalase II are not detectable in whole cells that have been subjected to an osmotic shock procedure (30), and the activities are present in the shock supernatant (Rutherford & Cooper, unpublished observations). This suggests that the enzymes may be located in the periplasmic space.

The only report to date of an organism defective in glyoxalase I activity appeared very recently (61). It was proposed that *S. cerevisiae* catabolizes glycerol in an unregulated fashion, and measurement of metabolite concentrations showed that glyceraldehyde 3-phosphate rises under such conditions. Surprisingly, the concentration of dihydroxyacetone phosphate was hardly affected.

Nevertheless, methylglyoxal was formed nonenzymatically in cells challenged with glycerol, and it was suggested that the glyoxalase enzymes played a significant role in removing this toxic metabolite. In fact the rationale for isolating a glyoxalase-defective mutant assumed that such a strain would be unable to grow on glycerol because any methylglyoxal formed from triosephosphates would not be transformed to harmless metabolites. That a glyoxalase I–negative mutant was obtained strongly supports the proposal that in *S. cerevisiae* glyoxalase I functions in the detoxification of methylglyoxal.

In fact, the mutant was killed by exposure to glycerol. This further suggests that *S. cerevisiae* has no other enzyme that could transform methylglyoxal into harmless metabolites, a significant point in relation to formaldehyde dehydrogenase that will be considered later.

Methylglyoxal Dehydrogenase

An enzyme able to oxidize methylglyoxal to give pyruvate was present in a pseudomonad after growth on aminoacetone (32). The K_m for methylglyoxal was low (30 μM), but although the enzyme appeared to be inducible, its activity was also low. In this particular organism the glyoxalase enzymes were present and at 30-fold the activity of the methylglyoxal dehydrogenase.

A quite different situation exists for certain gram-positive organisms growing on acetone or isopropanol (79). They showed no glyoxalase activity but possessed methylglyoxal dehydrogenase at high activity. The enzyme used NAD^+ as cosubstrate and was induced by growth on acetone. It seemed that the only significant transformation of methylglyoxal in these organisms was its direct oxidation to pyruvate. However, no further characterization of this interesting enzyme has yet been reported.

Formaldehyde Dehydrogenase

An unusual feature of formaldehyde dehydrogenase is that it can accept methylglyoxal as a substrate even though it is inactive against acetaldehyde (70). The enzyme from yeast and many other sources requires glutathione for activity and is believed to be active against the adduct formed between formaldehyde and glutathione.

The NAD^+-dependent oxidation of this adduct produces formylglutathione, which in turn is hydrolyzed by *S*-formylglutathione hydrolase (EC 3.1.2.12) to give formate and glutathione in a reaction reminiscent of that catalyzed by glyoxalase II. The oxidation of the methylglyoxal-glutathione adduct proceeds readily and presumably produces pyruvylglutathione. Hydrolysis of this compound would produce pyruvate and glutathione. However, there is some indication that *S*-formylglutathione hydrolase is without action on pyruvylglutathione (52, 82), so the significance of formaldehyde dehydrogenase in methylglyoxal utilization remains unclear.

OVERPRODUCTION OF METHYLGLYOXAL

The cytotoxic nature of methylglyoxal suggests that cells making this compound need to keep its concentration under strict control. If it is produced at a rate greater than its rate of utilization the surplus will accumulate in the cells and growth medium and would be expected to cause growth inhibition. The regulation of methylglyoxal synthase activity already described suggests a mechanism for the control of formation, but no regulation of utilization has yet been reported.

It seems likely that if the normal controls do not operate, either as a result of mutation or as a consequence of physiological alterations, cells could produce methylglyoxal to such an extent that they are killed.

Mutations That Permit Methylglyoxal Overproduction

One of the earliest reports of methylglyoxal overproduction involved a triosephosphate isomerase–negative mutant grown on gluconate and then exposed to glycerol (14). Under such conditions the dihydroxyacetone phosphate formed from glycerol could not be isomerized to glyceraldehyde 3-phosphate, and this in addition would preclude the formation of fructose 1,6-bisphosphate, the regulator of glycerol kinase activity (92). As a consequence of the metabolic block and the unregulated glycerol phosphorylation, the internal dihydroxyacetone phosphate concentration would rise and lead to activation of methylglyoxal synthase.

The triosephosphate isomerase–negative mutant also produced methylglyoxal at 0.3 mM concentration in the growth medium during catabolism of glucose. Under these conditions fructose 1,6-bisphosphate cleavage produces equimolar amounts of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, but only the latter can be further transformed by the glycolytic reactions. A double mutant that lacked both triosephosphate isomerase and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) behaved similarly.

In this double mutant, glucose could be catabolized only by the glycolytic pathway. When cells previously grown on gluconate were transferred to glucose medium they grew with a doubling time of 4–5 hr, and methylglyoxal was again present at 0.3 mM concentration in the growth medium. After 3–4 cell doublings, when all the glucose had been used, neither methylglyoxal nor lactate could be detected in the medium. This implied that essentially all the dihydroxyacetone phosphate formed from glucose had been catabolized, presumably via methylglyoxal and lactate.

Despite this clear ability to catabolize glucose, triosephosphate isomerase–negative mutants were unable to grow on glucose when inoculated onto solid media. The reason for this inability to grow persistently on glucose was unclear, because no metabolite became unavailable as a result of the mutation.

It seems likely that at very low cell densities the methylglyoxal produced is totally inhibitory.

A striking case of methylglyoxal overproduction came from studies of a double mutant constitutive for the glycerol catabolic enzymes and with a glycerol kinase insensitive to feed-back inhibition by fructose 1,6-bisphosphate. This organism grew, apparently normally, on glycerol where self-catabolite repression modulated the activity of the glycerol catabolic enzymes. However, during growth on succinate the enzymes were fully expressed, and such cells, challenged with glycerol, catabolized the glycerol very rapidly. Methylglyoxal was produced such that the concentration reached 0.8–1.4 mM in the growth medium, and the cells were killed (25).

A mutant also constitutive for the glycerol catabolic enzymes but with normal fructose 1,6-bisphosphate regulation of glycerol kinase did not produce lethal amounts of methylglyoxal under the same conditions. It seems likely that methylglyoxal overproduction was due to rapid, unregulated, glycerol phosphorylation leading to a significant increase in the internal dihydroxyacetone phosphate concentration, thereby activating methylglyoxal synthase.

A similar situation has been reported for a mutant of *E. coli* constitutive for the gluconate catabolic enzymes (66). When cells were grown on casein hydrolysate, the gluconate catabolic enzymes were not repressed, and addition of gluconate arrested their growth. Rapid catabolism of gluconate was necessary for this effect because the gluconate catabolic enzymes were repressed in cells grown on glucose, and such cells were not affected by the addition of gluconate. The inhibition was due to overproduction of methylglyoxal, which reached a concentration of 1.1 mM in the growth medium.

Because glyceraldehyde 3-phosphate and pyruvate are the immediate end products of gluconate catabolism by the Entner-Doudoroff pathway, it was supposed that the rapid metabolism of gluconate caused a rise in the glyceraldehyde 3-phosphate concentration and hence in dihydroxyacetone phosphate concentration, through the action of triosephosphate isomerase.

These effects of glycerol and gluconate appear to be a consequence of interference with the normal mechanisms that regulate metabolite flow at the initial stages of catabolism. When such defenses are breached the control of the methylglyoxal synthase reaction becomes vulnerable.

Cyclic AMP Stimulation of Methylglyoxal Production

The addition of 3',5'-cyclic AMP (cAMP) to wild-type cells growing on xylose, L-arabinose, or glucose 6-phosphate resulted in the accumulation of methylglyoxal in the growth medium at 1 mM concentration or greater, and the cells were killed (1). These findings were confirmed and extended because the presence of increased glyoxylase I activity prevented this lethal accumulation of methylglyoxal (12). With xylose it seemed likely that the effect of cAMP

was to activate an early step in catabolism, possibly permeation, such that an increase in dihydroxyacetone phosphate occurred with consequent activation of methylglyoxal synthase (1).

Support for this came from the observation that a mutant with a temperature-sensitive fructose bisphosphate aldolase (in which the activity of the aldolase may be rate-limiting for growth even at the permissive temperature) failed to produce an excess of methylglyoxal when challenged with cAMP during growth on glucose 6-phosphate at 30°C (12).

Growth arrest due to overproduction of methylglyoxal from xylose caused by cAMP occurred even if other carbon sources were present. By selection on plates containing cAMP, xylose, and glycerol, mutants that were resistant to growth inhibition were readily obtained (1). Such mutants did not produce methylglyoxal during growth on xylose in the presence of cAMP but were just as sensitive to the action of exogenous methylglyoxal as the wild-type cells. In addition, the mutation did not prevent overproduction of methylglyoxal caused by cAMP during growth on L-arabinose or glucose 6-phosphate.

The locus involved was termed *cxm* (for *c*AMP, *x*ylose, *m*ethylglyoxal production) and was found to map between *proA* and *lac*, near min. 7 on the *E. coli* genetic map (1). Deletions that removed this region of the chromosome result in the Cxm phenotype, indicating that the property is due to the absence of a functional product of the *cxm* gene but the nature of this product is still unknown.

The three sugars just described are some of those sugars whose transport into *E. coli* cells does not involve the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The stimulation of excessive methylglyoxal production by cAMP during growth of *Proteus mirabilis* on three other non-PTS sugars, glycerol, galactose, and trehalose, has been reported (71), suggesting that the cAMP effect may occur generally with non-PTS sugars.

Evidence in support of this has been presented as a result of studies with *E. coli* mutants defective in adenylate cyclase. Such mutants are unable to grow on carbon sources where enzyme induction is necessary (59), but secondary mutants that had overcome this deficiency were isolated. These secondary mutants, termed *csm* (*c*AMP *s*uppressor *m*utation), still carried the mutation in adenylate cyclase and the new mutation mapped in or near the *crp* gene.

Growth of the *csm* mutants on a variety of sugars was inhibited by exogenous cAMP, and in the case of the non-PTS sugars xylose, L-arabinose, galactose, and melibiose, the inhibition was due to excessive production of methylglyoxal (47). The biochemical reason for the excessive production of methylglyoxal is unknown, and the situation is obviously complex, because no methylglyoxal was produced when the same mutants were inhibited by cAMP during growth on sugars transported by the PTS.

There have been reports of the production of inhibitory concentrations of methylglyoxal in the absence of added cAMP by mutants growing on glucose 6-phosphate (12, 63). In one case the mutation responsible appeared to be in the *crp* gene (63). Assay of a variety of enzymes showed the only significant difference between the mutant and its parent was in the activity of phosphofructokinase (EC 2.7.1.11).

The mutant showed a 22% increase in activity; since it was proposed that phosphofructokinase was the rate-limiting reaction for glucose 6-phosphate catabolism, this increase was deemed to be significant in terms of increased triosephosphate formation (63). The situation seems complex: in contrast to earlier findings (1, 12), the parental organism was not inhibited by exogenous cAMP during growth on glucose 6-phosphate.

Other Causes of Methylglyoxal Overproduction

A curious aspect of self inhibition by methylglyoxal has been reported recently (60). Cells of *Streptococcus faecalis* that had been heated at 60°C for 21 min produced methylglyoxal to concentrations of about 1 mM when they were subsequently resuspended at 33°C in no-growth medium that contained glucose. Unheated cells did not form methylglyoxal under the same conditions.

It is not clear how the methylglyoxal was produced, because methylglyoxal synthase has not yet been found in *S. faecalis*. It is possible that the enzyme is present and that heat treatment alters conditions such that the dihydroxyacetone phosphate concentration is raised, allowing methylglyoxal to be produced. Alternatively, heating the cells may activate an otherwise inactive methylglyoxal-producing enzyme or destroy enzymes normally responsible for the further metabolism of methylglyoxal, thereby causing it to accumulate.

PERSPECTIVE

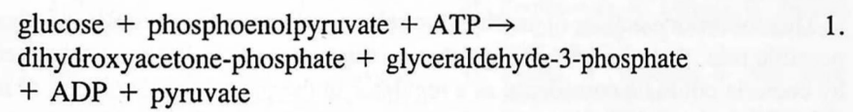
In many ways, methylglyoxal is still an enigma. Its only clear role to date is as an intermediate in isopropanol and acetone degradation by certain gram-positive bacteria (79). Its role in *E. coli* metabolism is still a matter for conjecture, but to quote from F. G. Hopkins (34), "we find that some few still hold that in the case of an agent separated from a living system, and studied in vitro, the assumption that it has a function in life partakes of the teleological. It seems more logical to believe that during evolution a functionless agent would have been eliminated." So some speculation on the possible roles of methylglyoxal is worthwhile.

One possible function is in the uncoupling of catabolism and anabolism (29). Methylglyoxal synthase can be viewed as providing an alternative to the glyceraldehyde 3-phosphate dehydrogenase reaction for the catabolism of triosephosphates. The measured maximal activities of methylglyoxal synthase

and glyceraldehyde 3-phosphate dehydrogenase in crude extracts of *E. coli* are equal (17, 35), but for both enzymes the limiting factor in vivo may be the availability of substrate. The normal cellular concentration of dihydroxyacetone phosphate in *E. coli* was found to be 0.19–0.24 mM (45, 50), about half the reported K_m value for methylglyoxal synthase (36). Glyceraldehyde 3-phosphate dehydrogenase has a K_m for Pi of 0.29 mM (6), so it would seem likely that under normal circumstances where glyceraldehyde 3-phosphate dehydrogenase can function, the Pi concentration would be sufficient to make the methylglyoxal synthase essentially inactive.

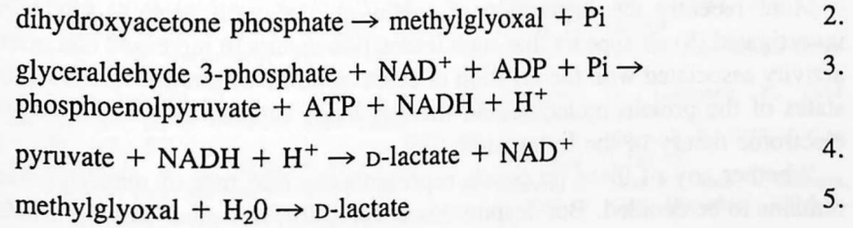
The conditions necessary for active methylglyoxal synthase would be a high concentration of dihydroxyacetone phosphate or a low concentration of Pi. Changes that resulted in a rise in the dihydroxyacetone phosphate concentration or a drop in the Pi concentration would permit triosephosphates to be catabolized via methylglyoxal synthase; otherwise they would follow the Embden-Meyerhof sequence.

In the natural environment of *E. coli*, Pi may not always be available and may be limiting for growth. The absence of Pi would prevent the operation of the glyceraldehyde 3-phosphate dehydrogenase reaction and so lead to a block in glycolysis. Thus a molecule of glucose would be transformed only as far as the triosephosphates, consuming a molecule of phosphoenolpyruvate (for transport by the PTS) and a molecule of ATP in the process:



glucose + phosphoenolpyruvate + ATP → dihydroxyacetone-phosphate + glyceraldehyde-3-phosphate + ADP + pyruvate (1.)

Catabolism of the dihydroxyacetone phosphate by methylglyoxal synthase would produce Pi necessary for the operation of the glyceraldehyde 3-phosphate dehydrogenase reaction, and glucose catabolism would then be possible:



dihydroxyacetone phosphate → methylglyoxal + Pi (2.)

glyceraldehyde 3-phosphate + NAD^+ + ADP + Pi → phosphoenolpyruvate + ATP + NADH + H^+ (3.)

pyruvate + NADH + H^+ → D-lactate + NAD^+ (4.)

methylglyoxal + H_2O → D-lactate (5.)

By all these reactions a molecule of glucose would be transformed to two molecules of D-lactate, and the reactions would proceed with no net synthesis of ATP. With no overall ATP yield such relations would not permit cell growth,

but they would allow glucose to continue to be broken down. It has been known for many years that the rate of glucose utilization by nongrowing *E. coli* is about 50% of that of growing cells (69). In such circumstances either operation of an ATPase or involvement of methylglyoxal as described above may be necessary to permit glucose catabolism to proceed.

An alternative role of methylglyoxal may be as a precursor of D-lactate. This latter substance can be used under aerobic conditions to energize the *E. coli* cell membrane such that the transport of various sugars and amino acids can proceed (39). *E. coli* can form D-lactate through the operation of an NAD^+-linked lactate dehydrogenase (EC 1.1.1.28), but this enzyme is thought to be mainly an enzyme of anaerobic metabolism and is optimally active only at high pyruvate concentrations (78).

Although methylglyoxal synthase is present in the cytoplasm, the glyoxalase system is apparently situated in the periplasmic space and so would form D-lactate from methylglyoxal outside the cytoplasmic membrane. If the flavin-linked D-lactate dehydrogenase is located on the outer surface of the cytoplasmic membrane, external D-lactate would be required for membrane energization. It may be significant that cAMP stimulates methylglyoxal production only with sugars that are not transported by the phosphotransferase system. In this situation cAMP, in stimulating methylglyoxal formation, also stimulates D-lactate formation in circumstances where an energized membrane is needed for transport or for ATP formation.

The inhibitory action of methylglyoxal on cell growth suggests another possible role, that of a growth regulator. In this sense methylglyoxal produced by bacteria could be considered as a regulator in the producing organism or in any cell that the methylglyoxal comes into contact with. A simple theory of cell-growth regulation involving the antagonistic action of a growth-promoting substance (promine) and a growth-inhibiting substance (retine) had been proposed (19) before the enzymatic formation of methylglyoxal was described. It was suggested that the glyoxalase system acted as promine by removing a growth-inhibiting α-ketoaldehyde, most likely methylglyoxal.

More recently the interaction of methylglyoxal with proteins has been investigated (8). It appears that such interaction results in increased electronic activity associated with the creation of electron holes within the valency band states of the protein molecule and thereby lends support to Szent-György's electronic theory of the living state (76).

Whether any of these proposals represents the true role of methylglyoxal remains to be decided. But despite this uncertainty, it is clear that significant progress has been made in the last decade. With its enzymatic formation now firmly established, it should not take another 50 years to determine the proper place of methylglyoxal in cell metabolism.

Literature Cited

1. Ackerman, R. S., Cozzarelli, N. R., Epstein, W. 1974. *J. Bacteriol.* 119:357–62
2. Anderson, A., Cooper, R. A. 1969. *FEBS Lett.* 4:19–20
3. Apontoweil, P., Berends, W. 1975. *Biochim. Biophys. Acta* 399:10–22
4. Babel, W., Hofmann, K. H. 1981. *FEMS Microbiol. Lett.* 10:133–36
5. Baumann, P., Baumann, L. 1975. *Arch. Microbiol.* 105:225–40
6. Bergmeyer, H. U., ed. 1974. *Methods of Enzymatic Analysis*, p. 466. Weinheim. New York. Verlag Chemie. Academic. 2302 pp. 2nd Eng. ed.
7. Böck, A., Neidhardt, F. C. 1966. *J. Bacteriol.* 92:470–76
8. Bone, S., Lewis, T. J., Pethig, R., Szent-György, A. 1978. *Proc. Natl. Acad. Sci. USA* 75:315–18
9. Branlant, C., Krol, A., Machatt, A., Ebel, J.-P. 1981. *Nucleic Acids Res.* 9:293–307
10. Carbon, P., Ehresmann, C., Ehresmann, B., Ebel, J.-P. 1978. *FEBS Lett.* 94:152–56
11. Cooper, R. A. 1974. *Eur. J. Biochem.* 44:81–86
12. Cooper, R. A. 1975. *Biochem. Soc. Trans.* 3:837–40
13. Cooper, R. A. 1975. *Meth. Enzymol.* 41B:502–8
14. Cooper, R. A., Anderson, A. 1970. *FEBS Lett.* 11:273–76
15. Cozzarelli, N. R., Koch, J. P., Hayashi, S., Lin, E. C. C. 1965. *J. Bacteriol.* 90:1325–29
16. Dakin, H. D., Dudley, H. W. 1913. *J. Biol. Chem.* 14:423–31
17. D'Alessio, G., Josse, J. 1971. *J. Biol. Chem.* 246:4319–25
18. Együd, L. G., Szent-György, A. 1966. *Proc. Natl. Acad. Sci. USA* 55:388–93
19. Együd, L. G., Szent-Györgyi, A. 1966. *Proc. Natl. Acad. Sci. USA* 56:203–7
20. Elliot, W. H. 1959. *Nature* 183:1051–52
21. Embden, G., Deuticke, H. J., Kraft, G. 1933. *Klin. Wochenschr.* 12:213–15
22. Entner, N., Doudoroff, M. 1952. *J. Biol. Chem.* 196:853–62
23. Fraenkel, D. G. 1968. *J. Biol. Chem.* 243:6451–57
24. Fraval, H. N. A., McBrien, D. C. H. 1980. *J. Gen. Microbiol.* 117:127–34
25. Freedberg, W. B., Kistler, W. S., Lin, E. C. C. 1971. *J. Bacteriol.* 108:137–44
26. Fromageot, C. 1929. *Biochem. Z.* 216:467–74
27. Garrido-Pertierra, A., Cooper, R. A. 1977. *J. Bacteriol.* 129:1208–14
28. Green, M. L., Lewis, J. B. 1968. *Biochem. J.* 106:267–70
29. Gunsalus, I. C., Shuster, C. W. 1961. In *The Bacteria*, ed. I. C. Gunsalus, R. Y. Stanier, 2:1–58. New York: Academic. 572 pp.
30. Heppel, L. A. 1968. *Meth. Enzymol.* 12B:841–46
31. Higgins, I. J., Turner, J. M. 1966. *Biochem. J.* 99:26P
32. Higgins, I. J., Turner, J. M. 1969. *Biochim. Biophys. Acta* 184:464–67
33. Hillman, J. D., Fraenkel, D. G. 1975. *J. Bacteriol.* 122:1175–79
34. Hopkins, F. G., Morgan, E. J. 1945. *Biochem. J.* 39:320–24
35. Hopper, D. J., Cooper, R. A. 1971. *FEBS Lett.* 13:213–16
36. Hopper, D. J., Cooper, R. A. 1972. *Biochem. J.* 128:321–29
37. Irani, M., Maitra, P. K. 1974. *Biochem. Biophys. Res. Commun.* 56:127–33
38. Iyengar, R., Rose, I. A. 1983. *J. Am. Chem. Soc.* 105:3301–3
39. Kaback, H. R. 1974. *Science* 186:882–92
40. Kline, E. S., Mahler, H. R. 1965. *Ann. N.Y. Acad. Sci.* 119:905–19
41. Kozarich, J. W., Deegan, J. L. 1979. *J. Biol. Chem.* 254:9345–48
42. Krymkiewicz, N., Diéguez, E., Rekarte, U. D., Zwaig, N. 1971. *J. Bacteriol.* 108:1338–47
43. Kundig, W., Roseman, S. 1966. *Meth. Enzymol.* 9:396–403
44. Kuroya, M. 1931. *Biochem. Z.* 235:438–43
45. Lowry, O. H., Carter, J., Ward, J. B., Glaser, L. 1971. *J. Biol. Chem.* 246:6511–21
46. Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixson, W. S., Crawford, E. J. 1954. *J. Biol. Chem.* 207:19–37
47. Melton, T., Snow, L. L., Freitag, C. S., Dobrogosz, W. J. 1981. *Mol. Gen. Genet.* 182:480–89
48. Meyerhof, O. 1948. *Experientia* 4:169–76
49. Meyerhof, O., Lohmann, K. 1934. *Biochem. Z.* 271:89–110
50. Moses, V., Sharp, P. B. 1972. *J. Gen. Microbiol.* 71:181–90
51. Murata, K., Tani, K., Kato, J., Chibata, I. 1980. *J. Gen. Microbiol.* 120:545–47
52. Neben, I., Sahm, H., Kula, M.-R. 1980. *Biochim. Biophys. Acta* 614:81–91
53. Negelein, E., Brömel, H. 1939. *Biochem. Z.* 303:132–44

54. Neuberg, C. 1913. *Biochem. Z.* 49:502–6
55. Neuberg, C., Gorr, G. 1925. *Biochem. Z.* 162:490–95
56. Neuberg, C., Kobel, M. 1930. *Biochem. Z.* 229:433–42
57. Neuberg, C., Scheuer, M. 1930. *Ber. Drsch. Chem. Ges.* 63B:3068–72
58. Otsuka, H., Együd, L. G. 1968. *Curr. in Mod. Biol.* 2:106–10
59. Pastan, I., Perlman, R. 1970. *Science* 169:339–44
60. Payne, J., Hilton, M. G., Gooch, J. E. T. 1981. *Can. J. Microbiol.* 27:65–71
61. Penninckx, M. J., Jaspers, C. J., Legrain, M. J. 1983. *J. Biol. Chem.* 258:6030–36
62. Pett, L. B., Wynne, A. M. 1932. *J. Biol. Chem.* 97:177–82
63. Puskas, R., Fredd, N., Gazdar, C., Peterkofsky, A. 1983. *Arch. Biochem. Biophys.* 223:503–13
64. Racker, E. 1951. *J. Biol. Chem.* 190:685–96
65. Racker, E. 1955. *Meth. Enzymol.* 1:454–58
66. Rekarte, U. D., Zwaig, N., Istúriz, T. 1973. *J. Bacteriol.* 115:727–31
67. Riddle, V., Lorenz, F. W. 1968. *J. Biol. Chem.* 243:2718–24
68. Rizza, V., Hu, A. S. L. 1973. *Biochem. Biophys. Res. Commun.* 54:168–75
69. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., Britten, R. J. 1963. *Studies of Biosynthesis in E. coli* 424–28. Wash. DC: Carnegie Inst. 521 pp.
70. Schütte, H., Kula, M.-R., Sahm, H. 1982. *Meth. Enzymol.* 89:527–31
71. Schwarzhoff, R. H., Williams, F. D. 1976. *J. Bacteriol.* 127:1590–92
72. Sibley, J., Lehninger, A. 1947. *J. Biol. Chem.* 177:859–72
73. Still, J. L. 1941. *Biochem. J.* 35:390–91
74. Summers, M. C., Rose I. A. 1977. *J. Am. Chem. Soc.* 99:4475–78
75. Suthers, A. J., Walker, T. K. 1932. *Biochem. J.* 26:317–22
76. Szent-György, A. 1977. *Proc. Natl. Acad. Sci. USA* 74:2844–47
77. Tanabe Seiyaku Co. Ltd. 1981. *Kokai Tokkyo Koho (Jpn. Patent)* 81 82, 099
78. Tarmy, E. M., Kaplan, N. O. 1968. *J. Biol. Chem.* 243:2587–96
79. Taylor, D. G., Trudgill, P. W., Cripps, R. E., Harris, P. R. 1980. *J. Gen. Microbiol.* 118:159–70
80. Toenniessen, E., Fischer, W. 1926. *Hoppe-Seyler's Z. Physiol. Chem.* 161:254–64
81. Tsai, P.-K., Gracy, R. W. 1976. *J. Biol. Chem.* 251:364–67
82. Uotila, L., Koivusalo, M. 1974. *J. Biol. Chem.* 249:7664–72
83. Vander Jagt, D. L., Han, L.-P. B., Lehman, C. H. 1972. *Biochemistry* 11:3735–40
84. Vella, G. J., Hill, R. E., Spenser, I. D. 1981. *J. Biol. Chem.* 256:10469–74
85. Wang, S.-L., Chen, J.-P., Shen, S.-C. 1964. *Scientia Sinica* 13:167–68
86. Werkman, C. H., Stone, R. W., Wood, H. G. 1937. *J. Bacteriol.* 33:100–101
87. Widmann, E. 1929. *Biochem. Z.* 216: 475–78
88. Yamasaki, I. 1930. *Biochem. Z.* 218: 468–71
89. Yuan, P. M., Gracy, R. W., Hartman, F. C. 1977. *Biochem. Biophys. Res. Commun.* 74:1007–13
90. Yuan, P.-M., Tsai, P. K., Gracy, R. W. 1975. *18th West Central States Biochem. Conf.* Stillwater, Ok. p. 15 (Abstr.)
91. Zwaig, N., Diéguez, E. 1970. *Biochem. Biophys. Res. Commun.* 40:1415–22
92. Zwaig, N., Lin, E. C. C. 1966. *Science* 153:755–57

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THE RELATIONSHIP OF PLASMID-MEDIATED IRON TRANSPORT AND BACTERIAL VIRULENCE

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INTRODUCTION

The iron content of human plasma is high—about 20 μM—but the amount of free iron is on the order of 10^{-18} M (21, 24). This extremely low concentration of free iron is insufficient for bacterial growth and is due to the presence of high-affinity iron-binding proteins like transferrin in serum and lactoferrin in secretions (21–23). Pathogens attempting to establish an infection must, therefore, be able to adapt to this iron-limited environment. Thus, a mechanism whereby pathogenic bacteria can utilize the otherwise unavailable iron could be an important contributor to their ability to cause disease. It is my intention to first review the earlier findings linking iron transport and virulence in different bacterial systems. I will then turn to the analysis of the two bacterial plasmids,

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pJM1 in *Vibrio anguillarum* and pColV-K30 in *Escherichia coli*, that have recently been demonstrated to code for iron-transport systems associated with the virulence of these bacteria.

IRON AND PATHOGENESIS

About half a century ago, Waring & Werkman (177) demonstrated that bacteria require iron for growth. At about the same time, Shade & Caroline (149) reported the presence of iron-binding proteins in serum, which, in their view, could confer serum antibacterial activity.

In 1956, Jackson & Burrows (82) observed that wild-type *Yersinia pestis* could utilize exogenous hemin on solid medium and that "nonpigmented" mutants incapable of accumulating the compound on their surface were avirulent. It was then described (117) that bacteria possess low-molecular-weight, iron-binding compounds or siderophores that enable them to utilize the iron they require for growth. Some of these siderophores may successfully compete and acquire iron from the serum iron-binding proteins (183, 185). Wake et al (174) showed a correlation between siderophore production and virulence in *Y. pestis*.

In the case of *Y. pestis* it was later shown by Perry & Brubaker (130) that hemin could serve all *Yersiniae* as a sole source of iron. In the initial study (82), nonpigmented mutants were phenotipically restored to virulence in mice receiving a concomitant injection of sufficient iron to saturate serum transferrin. Payne & Finkelstein (127, 129) determined that the virulence of strains of *Neisseria gonorrhoeae* to chick embryos was enhanced by the addition of iron.

The actual role of injected iron has not really been defined with certainty. The most popular notion is that injected iron simply fulfills a nutritional requirement. Alternatively, injected iron may interfere with one or more mechanisms of host defense (85). Of course, injection of sufficient iron to interfere with host defenses would almost surely supply the invading bacteria with more than enough iron for growth.

Nevertheless, it was recently determined that an avirulent phenotype of *Y. pestis*, not thought to be blocked in assimilation of iron, can be restored to full virulence in mice by concomitant injection of sufficient iron to saturate serum transferrin (18). It is of interest that this mutant has lost the pesticin plasmid (44) that also mediates coagulase and fibrinolytic activities (3) now thought to reside on the outer membrane (162). Curiously, pesticin itself appears to absorb to the hemin receptor of *Y. pestis* (17), a situation analogous to the absorbtion of bacteriocins to receptors for siderophores (119), although resistance or tolerance to pesticin did not involve the loss of receptors for known siderophores (45). This observation was anticipated because the *Yersiniae* were the first invasive pathogens shown to accumulate iron via siderophore-independent processes (130).

More recent work also suggested the existence of siderophore-independent iron-uptake systems in members of the genus *Neisseria* (121). All isolates of *N. meningitidis* can utilize the iron bound to transferrin by a direct contact between a membrane receptor and the iron-transferrin molecule (2, 151). A large number of gonococcal isolates and all meningococci had also the ability to use lactoferrin-bound as well as transferrin-bound iron (109, 110). Direct contact of lactoferrin and bacterial membranes was demonstrated for *N. gonorrhoeae* (111). The characterization of iron-regulated outer membrane proteins was also reported (120).

Other investigators (46, 194, 195) have detected siderophores in culture supernatants of *Neisseria spp.* that permit the growth of *N. meningitidis* and *N. gonorrhoeae* in iron-restricted environments. However, the detection of these siderophores is now complicated by the fact that a siderophore-like factor has recently been isolated after processing large quantities of control culture medium (C. Sciortino and R. A. Finkelstein, personal communication).

Evidence has now accumulated, however, indicating that most pathogens obtain iron by producing siderophores, which can successfully compete with the iron-binding proteins of the host for the otherwise unavailable iron. Siderophores are broadly classified chemically into two classes: phenolates and hydroxamates (118, 119). The role of siderophores in the virulence of bacterial pathogens has been determined for only a few cases.

One of the best-studied siderophores is enterobactin, a phenolate siderophore. This compound is produced by bacteria of the genera *Escherichia, Salmonella,* and *Klebsiella* (142) and has been identified as the cyclic trimer of 2,3-dihydroxybenzoyl serine. Rogers (141) has proposed that secretion of enterobactin is essential for in vivo bacterial growth, and Griffiths & Humphreys (62) found that this siderophore is produced in vivo during fatal infections with *E. coli*. The ability of *Salmonella typhimurium* to grow in human serum and to cause lethal infection of mice is absolutely dependent on the production of enterobactin (193) or 2,3-dihydroxy-benzoate (77), an enterobactin precursor. Several pathogenic vibrios were recently shown to produce siderophores, although chemical and biological assays indicated that several different iron transport systems are expressed by members of this genus (1, 128).

Phenolate compounds were detected in culture supernatants of iron-starved *V. cholerae, V. fluvialis, V. vulnificus,* and *V. anguillarum* but not *V. parahemolyticus* or *V. alginolyticus*. The *V. cholerae* phenolate siderophore, vibriobactin, was recently characterized as a catecholamide (65). Vibriobactin does not possess spermidine as the polyamine backbone of the molecule. It has instead the rare *N*-(3-aminopropyl)-1,3-diaminopropane, norspermidine (65). Iron-regulated outer-membrane proteins were recently reported in *V. cholerae* (150), although whether vibriobactin or any other siderophore binds to specific proteins is as yet unknown.

V. vulnificus synthesizes an additional, relatively species-specific compound (1). This unique siderophore has greater biological activity than the *V. vulnificus* phenolate siderophore and has some properties that identify it as a hydroxamate siderophore, although it fails to react strongly in the Csaky test; consequently, the chemical nature of this siderophore is still somewhat obscure (1). Simpson & Oliver (152) reported that, in addition to the production of phenolate and hydroxamate siderophores, *V. vulnificus* can use at least one other siderophore, Desferal (desferrioxamine B mesylate, Ciba Pharmaceutical, Summit, NJ), to alleviate iron limitation.

It was previously reported (192) that addition of Desferal reversed the bactericidal effects of human serum and decreased the 50% lethal dose of *V. vulnificus* for mice by a factor of about 10^6. The inability of *V. vulnificus* to grow in normal serum indicates that the siderophores cannot successfully compete for the transferrin-bound iron. Alternatively, some other factors present in normal serum may be playing a role in the final outcome of the competition between bacteria and host-serum iron.

Several other bacteria can produce siderophores. *Pseudomonas aeruginosa* produces iron chelators of both the phenolate and hydroxamate type (119). One of these, pyochelin, is a phenolic rather than hydroxamic siderophore, but unrelated to dihydroxybenzoate (29). Cox (28) recently demonstrated that this siderophore increases the growth and lethality of virulent *P. aeruginosa* strains but only increases the survival of avirulent bacteria.

The plant pathogen *Agrobacterium tumefaciens,* when grown in low-iron media, synthesizes the phenolate siderophore agrobactin as well as several membrane proteins (99). Analysis of mutants suggests that one of these membrane proteins may be the receptor for ferric agrobactin (99).

Transport of iron into *Corynebacterium diphteriae* also occurs by a high-affinity, active-transport system that may involve an as yet uncharacterized siderophore (144).

It is important to point out here that other bacterial virulence determinants have some sort of iron control. For instance, excess of iron has been shown to inhibit yields of diphteria toxin (100, 125), *Shigella dysenteriae* type I toxin (39, 169), and *Pseudomonas aeruginosa* toxin A (9, 10). In addition, in the case of *P. aeruginosa* and in contrast to *Corynebacterium diphteriae* and *S. dysenteriae,* excess iron is also deleterious on the yields of other extracellular products involved in virulence, like protease and elastase (9).

BACTERIAL IRON TRANSPORT

In this section I will discuss aspects related to bacterial iron transport, focusing especially on *E. coli*. A more detailed analysis of the subject of siderophores, iron transport, and bacterial virulence can be found in the reviews by Bullen

(21), Bullen, Rogers & Griffiths (24), Lankford (97), Neilands (118, 119), and Weinberg (184).

There are several stages for iron assimilation in bacteria: synthesis of the essential siderophores, their secretion out of the bacterial cell, and, of course, the subsequent recognition and internalization of the siderophore-iron complex. In the best-studied systems, *E. coli* and *S. typhimurium,* in addition to the synthesis and excretion of enterobactin (122, 134, 135) both organisms utilize iron complexed with ferrichrome, a siderophore excreted by the fungus *Ustilago sphaerogena* (41, 98, 101, 119, 134, 148, 180). *E. coli* can also utilize iron that has been chelated by citrate. Both organisms use iron in the absence of known siderophores (119). This last mechanism requires very high concentrations of iron, and transport is inhibited with nonassimilable chelators (96).

Enterobactin metabolism has been extensively studied in *E. coli*. Three enzymes coded for by the *ent*A, *ent*B, and *ent*C genes catalyze the conversion of chorismic acid to 2,3-dihydroxybenzoic acid, and enzymes coded for by the *ent*D, *ent*E, *ent*F, and *ent*G genes intervene in the condensation of 2,3dihydroxy benzoic acid and L-serine to enterobactin (56–58, 103, 189, 196). Enterobactin is excreted by the cells by an unknown mechanism (51).

In addition to the biosynthetic genes, two additional genes have been mapped at 13 minutes. These are the *fep* and *fes* genes. The activity of the *fes* gene product has been defined as an esterase that degrades enterobactin to 2,3-dihydroxybenzoylserine as a means of releasing the chelated iron (96, 122). The *fep* gene is the structural gene for the outer membrane receptor for ferric enterobactin. Strains with a mutation in *fep* are unable to transport the ferric enterobactin complex (30); they thus become iron-starved and as a consequence overproduce enterobactin. Membranes from *fep* mutants lack ferric-enterobactin binding ability (76). Mutants selected for their resistance to colicin B, *cbr* (139), or *feu* (70) were shown to be also blocked in enterobactin-mediated iron transport.

It was further determined that enterobactin prevented adsorption of the colicin molecule to the cell (66), leading to the conclusion that colicin B and enterobactin were competing for a specific receptor. Based on spheroplast studies, mutants were classified into two classes, *fep*A and *fep*B, such that *fep*A mutants, if stripped of their outer membranes, were able to transport ferric enterobactin (191). Spheroplasts of *fep*B mutants were still unable to transport ferric enterobactin and were designated as having a lesion in a gene corresponding to a cytoplasmic membrane component.

Recently (131), a collection of hybrid lambda phages carrying *EcoRI* fragments of the *E. coli* chromosome was used to complement *fep*A, *fep*B, and *ent*A mutants. The data provided genetic evidence for the existence of the *fep*B gene and suggested the gene order (*ent*D *fes fep*A) *ent*F, *fep*B, *ent*C, *ent*A,

*ent*B, *ent*E. Fragments of the *ent* gene cluster were recently transposed into the conjugative R-plasmid RP4 by using bacteriophage Mu (94). Two restriction fragments, one carrying *ent* (ABCEG) and the other carrying *fep*, were cloned in vitro using multicopy vectors and one of the *RP4:ent* plasmids as a source of DNA enriched in enterobactin-system genes. A third restriction fragment, carrying the three remaining genes *ent*D, *fes*, and *ent*F was cloned directly from the chromosome (94).

By using a combination of Tn*5* mutagenesis, subcloning of restriction fragments, and restriction mapping, the order of the genes was established as *ent*D, *fes*, *ent*F, *fep*, and *ent* CAGBE. The nine genes were shown to be distributed across 26 kilobases of DNA and are organized into at least six transcriptional units (95).

The growth of *E. coli* cells in low-iron media results in the appearance of four high-molecular-weight polypeptides. Outer membrane proteins of 83, 81, 78, and 74 kilodaltons (kd) in *E. coli* (80, 107, 167) and 82, 79, and 77 kd in *S. typhimurium* (4) are expressed as major proteins under conditions of iron limitation. The synthesis of these proteins was coordinately controlled under all conditions examined (86). Examination of *feu*B mutants (colicin B–resistant) confirmed that the 81-kd protein was absent and corresponded to the receptor for colicin B (70, 139) and for enterobactin (81).

The uptake of the iron siderophore ferrichrome requires at least two genes designated *ton*A (*fhu*A) and *fhu*B (73, 135, 190). The *ton*A gene product is an outer membrane protein and the *fhu*B product appears to reside in the cytoplasmic membrane. Recently, the *ton*A (*fhu*A) gene was found in a collection of Col E1–*E. coli* chromosome hybrid plasmids.

Expression of the cloned *ton*A gene was obtained by using an in vitro transcription-translation system (133). The *ton*A protein precursor synthesized in vitro was about 2 kd larger than the mature membrane protein, which is 78 kd (16). The TonA protein can be used as a receptor by colicin M, phages T1, T5, and 80, as well as by the antibiotic albomycin, an analogue of ferrichrome and other closely related siderophores (102, 119).

A citrate-dependent iron-transport system is induced when *E. coli* cells are grown in media containing citrate (51, 79, 142). Under these conditions, an additional membrane protein of 80,500 daltons appears in the outer membrane (79, 173). Recently, by mutant analysis, it was concluded that this protein, called FecA, is probably the membrane receptor for ferric citrate transport.

The Cir protein (colicins I and V receptor) in the outer membrane is also induced under conditions of iron limitation, but whether it has any function in iron transport is as yet unknown (88, 89).

E. coli mutants with an overproduction of the outer-membrane proteins and constitutive for all iron-uptake systems were recently isolated by Hantke (71). He used operon fusions between *ton*A(*fhu*A) and the *lac* operon. The genes

were designated *fur* for *f*erric *i*ron–*u*ptake *r*egulation. Whether these genes play a regulatory role is still under investigation, although a tentative map position has been recently obtained by crosses with different Hfr strains, in a region between *lac* and the origin of HfrR4 or between *tsx* and *pur*E (72).

The *ton*B product is necessary for the uptake of iron by all high-affinity iron-transport systems (119). Its function and location are still unclear, although it may be required for transport across the inner membrane (191) and/or for physical association of outer and inner membranes (181). Molecular cloning experiments indicated that the product of the *ton*B gene is a 36-kd polypeptide (138). By using a lambda transducing phage carrying the *ton*B gene, Plastow & Holland (132) showed enrichment of the inner membrane with a 40-kd protein, suggesting that the location of the Ton B protein must be in the cytoplasmic membrane.

In addition, functions assumed to be in the cytoplasmic membrane appear to also be required for each system: *fep*B for enterobactin (131, 191), *fhu*B for ferrichrome (190), and *fec*B for ferric citrate (79). The *ton*B gene is of interest because it is also necessary for vitamin B12 uptake. Mutants in yet another gene, *exb*B, show a strong decrease of the uptake of ferrichrome and vitamin B12 (74). Unlike *ton*B, *exb*B mutants do not affect ferric enterobactin or ferric citrate transport.

Recently (86), two new outer-membrane proteins, with a molecular weight of 90 and 25 kd respectively, were reported to be regulated by iron. The production of the 90-kd protein apparently requires a high concentration of free intracellular iron, and iron stress immediately inhibits its biosynthesis. These authors hypothesized that the 90-kd protein intervenes as a storage protein, with similar properties to those reported for ferritin synthesis in vertebrates.

It was of interest that a burst of synthesis of the 25-kd protein precedes the induction of the 83-, 81-, and 74-kd proteins, but the synthesis of the 25-kd protein returns to basal levels regardless of the subsequent iron availability. It remains to be determined what the relationship of these proteins is to bacterial iron metabolism.

Pathogenic strains of *E. coli* were recently shown to synthesize the 83-, 81-, 78-, and 74-kd proteins (64). It is worth noting that in all clinical strains of *E. coli* examined, these proteins were produced in larger quantities than in *E. coli* K12 (64). Recent work has now shown that some normal human sera and sera from mice and rabbits contain IgG antibodies against the iron-regulated outer-membrane proteins (60). These antibodies, together with antibodies against enterobactin (114, 115), may contribute to the inhibition of bacterial multiplication in the vertebrate host.

E. coli cells growing under conditions of iron limitation contain altered species of tRNA (19, 61, 108). Identical tRNA changes are found in *E. coli* recovered from the peritoneal cavities of lethally infected animals (63). These

alterations are due to the presence of an undermodification of the normally (under iron-proficiency conditions) hypermodified nucleoside 2-methylthio-N^6-(^{2} isopentenyl) adenosine in the tRNAs during growth under conditions of iron limitation (19). The chromatographically altered tRNA species lack the methylthio (ms^2) moiety and have lowered translational efficiencies (19).

As a consequence, there is a relief of transcription termination at certain operons at the aromatic amino acid biosynthetic pathway, which results in an increased expression of these operons under iron-restricted conditions (19, 20, 60). Consequently, this mechanism could play an important role in regulating the biosynthesis of enterobactin, because this siderophore is synthesized by way of a branch pathway from the aromatic amino acid biosynthetic pathway (142), and iron is an essential component of the first enzyme of the common pathway (105).

ASSOCIATION OF PLASMIDS, IRON TRANSPORT, AND VIRULENCE

A number of plasmids have been found to carry genetic determinants for bacterial virulence (40). The most well-known examples are the Ent plasmids of enterotoxigenic strains of *E. coli* (67, 68, 156, 161). These strains do not achieve their full pathogenic potential unless they are also able to colonize the small intestine effectively by means of protein pili, which allow the bacteria to adhere to the wall of the vertebrate small intestine. These adhesive pili are also often encoded by plasmids (40, 42, 124, 159, 160).

Plasmids have been associated with other virulence mechanisms. For instance, the genetic determinant for hemolysin synthesis and secretion is located either on transmissible plasmids or on the chromosome (5, 112, 155, 168, 182). A plasmid-specified major outer-membrane protein, the *tra*T gene product, was found to be responsible for plasmid-mediated resistance to the serum bactericidal activity in certain *E. coli* strains (113, 165). Serum resistance can also be encoded on Col V plasmids (see below).

Early genetic studies indicated that chromosomal genes were necessary for virulence in the genus *Shigella* (47, 48, 53). More recent work has shown that large plasmids are also necessary for virulence in both *S. sonnei* (91, 145, 146) and *S. flexneri* (147) as well as in the "shigella-like" enteroinvasive *E. coli* (69). It was also recently shown that components of invasiveness in *Yersinia pestis* and *Y. enterocolitica* may be plasmid-specified (52, 136, 137, 197).

A large plasmid of about 120 megadaltons (Mdal) in virulent strains of *Agrobacterium tumefaciens* confers on the bacterial cell properties that allow them to transform higher plant cells into tumor cells during infection (83, 170, 179). Production of exfoliative toxin and regulation of enterotoxin B synthesis are also mediated by plasmids in certain strains of *Staphylococcus aureus* (40).

In recent years the virulence of two bacteria belonging to two different ecological niches was demonstrated to be due to the presence of specific plasmid classes. *E. coli* causing bacteremias in humans and domestic animals harbor the Col V plasmid, which encodes the small antibacterial protein colicin V. It was also demonstrated that highly virulent strains of the marine fish pathogen *Vibrio anguillarum,* which cause a disease characterized as a hemorrhagic septicemia, carry the pJM1 plasmid.

Experiments in my laboratory with the pJM1 plasmid (31, 32) and by Williams with the pColV-K30 system (186, 187) demonstrated that these two plasmids coded for novel iron transport systems that were highly efficient in obtaining iron from complexes of iron-transferrin. I will describe in the next sections these two plasmid-mediated iron-uptake systems and their contribution to the bacterial virulence repertoire.

The pColV-K30 plasmid of Escherichia coli

E. coli strains responsible for generalized infections and death in livestock and humans usually harbor plasmids that enhance their virulence and that also code for colicin V (12, 49, 50, 55, 78, 153–155, 157, 158).

Elimination of the Col V plasmids from these strains invariably reduced their pathogenicity for experimental animals. Virulence was regained upon reintroduction of the Col V plasmids into the now plasmidless strains. However, there is no correlation between the production of colicin V and virulence, as demonstrated by Quackenbusch & Falkow (140). By using transposition mutagenesis, these investigators showed that insertions inactivating colicin V production did not affect the virulence phenotype of the bacterium.

Smith (153) reported on a genetic trait carried by certain Col V plasmids that was indeed associated with virulence. Smith found that nonimmune sera from a variety of animal sources did not affect strains harboring a Col V plasmid to the same extent as it did plasmidless strains, suggesting that this type of Col V plasmid carried genetic determinants for resistance to the bactericidal effects of the host vertebrate serum. One of such plasmids, pColV,I-K94, is a conjugative F-like plasmid (84, 104) specifying colicins V and I (116).

Recently, Binns et al (8) showed that a 24.3-kb *BamH1* restriction endonuclease fragment from pColV,I-K94, cloned in the vector pBR322 (11), greatly enhanced the virulence of *E. coli* for chickens. A 5.3-kb sequence within this fragment carried the genetic locus for the phenotype expressed as an *i*ncreased *s*urvival in *s*erum *(iss)*. In this same work, some pBR322 clones were obtained in which the cloned restriction endonuclease fragment carried the colicin V structural genes. Upon transformation into suitable *E. coli* recipients, these clones specified colicin V production but caused no enhancement of the strain's ability to survive in serum nor of its pathogenicity.

Recently, Clancy & Savage (27) have described what appears to be yet

another phenotype in certain Col V plasmids that may enhance bacterial virulence. Strains harboring these Col V plasmids adhere in vitro to mouse intestinal epithelium in two- to three-fold greater numbers than do isogenic strains without the plasmid. Reintroduction of the Col V plasmid in these strains greatly enhanced their ability to adhere to the epithelial cells in vitro. Moreover, strains carrying these Col V plasmids had pili, as shown by electron microscopy, that adsorbed male-specific phages, whereas the isogenic, plasmidless strains did not.

Some Col V plasmids, but not pColV,I-K94, can relieve the effect of iron limitation imposed on *E. coli* strains deficient in the biosynthesis of enterobactin. For instance, the pColV-K30 (186), the pRJ100 (163), and the pColV-K311 (13) plasmids were shown to be essential to allow the enterobactin-deficient bacteria to grow in media containing iron chelators like the iron-binding protein transferrin. These results suggested that these plasmids possessed novel iron-uptake systems.

In the case of the pColV-K30 system, Williams & George (187) also performed in vivo experiments in mice that indicated not only that plasmid-carrying strains were virulent but also that iron added to the inoculum enhanced the virulence of only the isogenic strains from which the pColV-K30 plasmid had been cured. Moreover, in those cases in which mixtures of both plasmid-carrying and plasmidless bacteria were injected, results suggested that, in conditions of iron availability, there was little or no selective advantage in the possession of the pColV-K30 plasmid.

E. coli K12 strain LG1315, which is deficient in the biosynthesis of enterobactin, was unable to grow under conditions of iron limitation unless it was harboring the pColV-K30 plasmid (186). Mutants of this strain were recently isolated by chemical mutagenesis; they were defective in iron uptake, due to mutations on the pColV-K30 plasmid (188). These mutants were considerably attenuated in their pathogenicity but were unchanged in colicin V production.

Conversely, mutants that were defective in colicin V synthesis were not affected in iron-uptake properties nor in virulence. Crossfeeding experiments between these different plasmid iron uptake–deficient mutants identified two plasmid-mediated components: a diffusible iron-chelating compound and a nondiffusible membrane-associated substance. These two components allowed transport of iron into the bacterial cell.

Recently, the nature of these two components was clarified. The soluble iron-chelating compound was determined to be a hydroxamate-type siderophore, in studies carried out with strains carrying the Col V plasmid pRJ100 (163) and in several clinical isolates (164). This plasmid-mediated iron-chelating agent has now been identified as aerobactin (178), a siderophore originally found in supernatants from iron-limited cultures of *Aerobacter aerogenes* (54). The nondiffusible component, a membrane receptor for the iron-aerobactin complex, was now identified as a 74-kd outer-membrane

protein (7, 59, 172). Competition experiments carried out by de Graaf's laboratory (123, 171, 172) demonstrated that this protein receptor also serves as the binding site for the bacteriocin cloacin, produced by *Enterobacter cloacae* DF13.

Recent work carried out by Neilands's laboratory showed that, although ferric aerobactin has a stability constant many orders of magnitude below that of ferric enterobactin, it is nonetheless equipped with structural properties that enhance its ability to remove iron from transferrin (90). These characteristics, as well as the presence of antibodies against enterobactin (114, 115) and the enterobactin receptor (60) in vertebrate serum, may contribute to its status as a virulence determinant in clinical isolates of *E. coli*.

Four chromosomal genes—*ton*A (*fhu*A), *fhu*B, *ton*B, and *exb*B—determine functions for aerobactin uptake, in addition to those coded for by the Col V plasmid (15). These chromosomally mediated functions are also required for uptake of ferrichrome and other hydroxamate siderophores (119). Recently, Braun and collaborators found that the genes *fhu*C,D,B were also required for uptake of ferric aerobactin. The *fhu*A gene product is apparently not directly involved in the transport process, but mutations in these gene exerted a polar effect on the expression of the linked *fhu*B, C, and D genes so that ferric aerobactin uptake was no longer functioning (15a).

By insertion of phage Mu d1 (Ap *lac*) into the plasmid pColV-K311, Braun & Burkhardt (14) placed the expression of the β-galactosidase gene under the control of the iron state of the cells. In *fur* mutants, with an impaired regulation of all iron supply systems studied so far (71), β-galactosidase was synthesized in high amounts independently of the cellular iron concentration, indicating that it was no longer regulated by the iron supply (14).

From these results Braun and collaborators suggested that there is an iron-controlled promoter on the pColV-K311 plasmid that is subject to regulation by the chromosomal *fur* gene, although the nature of this regulation is still unclear. In a more recent work, Braun and collaborators used insertion mutagenesis with both the phage Mu d1 (Ap *lac*) and the transposition sequence Tn*5* to show that the aerobactin genes in the plasmid pColV-K311 are transcribed from the locus that determines aerobactin synthesis to the gene encoding the ferric aerobactin outer-membrane receptor (15a).

Recently, the aerobactin-mediated iron-uptake system present in the pColV-K30 plasmid was cloned into the multicopy vector pPlac (6). The plasmid pABN1 is one of such recombinant derivatives containing the iron-uptake regions in a cloned 16.3-kb *HindIII* fragment of pColV-K30. The plasmid pABN1 was then used to study the expression of the iron-uptake system in *E. coli* BN3040. This strain was defective in enterobactin biosynthesis and also had a mutation in the *cir* genes, resulting in the absence of the Cir protein, which comigrates in gels with the 74-kd ferric-aerobactin receptor.

In this background, it was possible to study the expression of the aerobactin

receptor encoded by the pABN1 clone. Results indicated that the expression was comparable to that observed for the strain BN3040 carrying the whole pColV-K30 plasmid when the strains were grown under conditions of iron limitation. It was of interest that at high iron concentration, there was still some expression of the 74-kd aerobactin receptor in the strain containing the cloned derivative. This residual iron expression could be due to the high copy number of the cloning vector (6).

Production of aerobactin by strains containing pABN1 was also demonstrated. Subcloning of pABN1 DNA generated plasmids in which, even though a *lac* promoter present in the cloning vector was eliminated, aerobactin was still being synthesized. This suggests the presence of at least one specific promoter in the cloned sequence. One of these subclones, pABN5, obtained by cleavage with the restriction endonucleases *HindIII* and *EcoRI*, did not express the 74-kd protein. This suggests that the genes for the aerobactin receptor must overlap the *EcoRI* restriction site (6).

In concurrent work carried out in de Graaf's laboratory (93), it was demonstrated that the genes for the 74-kd receptor were included in a 6.5-kb *BamHI* pColV-K30 fragment cloned in pBR322. By using minicells, these investigators demonstrated that a recombinant plasmid, pSF8, carrying the 6.5-kb *BamHI* fragment, encodes not only the 74-kd receptor but also a 50-kd protein (92). Deletion derivatives of pSF8 were also described (92). One of them encoded only the 50-kd polypeptide. Cells carrying this deleted derivative were no longer sensitive to cloacin and were iron-uptake deficient. Cells carrying deleted derivatives of pSF8 coding for the 74-kd protein only could not take up ferric aerobactin but were still cloacin-sensitive.

According to these results, it appears that uptake of ferric aerobactin requires expression of both the 74- and 50-kd polypeptides. Since the 50-kd protein is most likely located in or at the cytoplasmic membrane (92), it possibly functions in binding or transport of ferric aerobactin at the cytoplasmic membrane level.

The Vibrio anguillarum plasmid pJM1

Vibriosis is a very serious infectious disease that affects marine fish, the etiological agent being *V. anguillarum* (25, 26, 43, 143). High- and low-virulence strains of this bacterium have been described (35). The high-virulence phenotype of strains isolated in the Pacific Northwest was demonstrated to be conferred by a 65-kb plasmid, designated pJM1 (34, 35). This plasmid codes for a very efficient iron-uptake system that permits bacteria to grow under iron-restricted conditions (31, 32).

In vivo experiments showed that possession of this plasmid was instrumental for bacteria to establish infections (34). This selective advantage was a function of the iron concentration of the sample injected into the animal, as was the case

for *E. coli* strains harboring the pColV-K30 plasmid (187). High iron allowed the proliferation of plasmidless avirulent strains, with little if any difference when compared to high-virulence strains carrying the pJM1 plasmid (31, 32).

The kinetics of the pJM1 plasmid–mediated iron-transport process was analyzed by testing the amount of radioactive iron incorporated into nongrowing *V. anguillarum* cells (33). Under iron limitation conditions, plasmidless cells did not incorporate appreciable amounts of radioactive iron. Conversely, cells harboring pJM1 incorporated radioactive iron at a high rate, confirming that the ability of plasmid-carrying cells to grow under iron-limitation conditions was due to an enhanced incorporation of iron. Plasmid-mediated incorporation of radioactive iron was inhibited considerably by the energy inhibitor KCN, indicating that it was largely due to iron uptake into the cell cytosol.

A small proportion of the radioactive iron incorporated by cells carrying the pJM1 plasmid was unaffected by KCN and is likely to be due to specific binding to the cell membrane, because the amount incorporated by plasmidless cells, even in the absence of KCN, was much lower (33). Concomitant with efficient plasmid-mediated iron uptake, there was an induction of the synthesis of two novel outer-membrane proteins: OM2, an 86-kd protein associated with the presence of the pJM1 plasmid, and OM3, a 79-kd protein of chromosomal origin (33). The kinetic and membrane protein findings suggested that the plasmid-mediated OM2 protein may be the actual receptor for a complex of iron with a putative plasmid-mediated siderophore (33).

The pJM1 plasmid-mediated iron uptake system of *V. anguillarum* was further characterized by using the mutagenic capability of the transposition sequence Tn*1*. The R-plasmid RP4 (75) was used as the donor of Tn*1*, generating a series of iron uptake–proficient and –deficient derivatives in which Tn*1* was inserted at different sites on the pJM1 plasmid (36, 37, 176). In some cases, the inserted Tn*1* sequences generated deletions beginning in regions adjacent to the site of insertion.

The Tn*1*-generated, iron uptake–deficient mutants were grouped into two classes. One class consisted of those mutants that were able to be crossfed only by supernatant fluids from wild-type plasmid-carrying strains and could not crossfeed members of either class. Members of this class were most likely affected in genes responsible for the biosynthesis or activity of a putative plasmid-mediated siderophore. Members of the second class, which could not be crossfed by supernatants from wild-type strains, had not only a lesion in genes involved in siderophore production or activity but also in DNA regions associated with the biosynthesis or activity of a membrane receptor for the iron-siderophore complex. Restriction endonuclease cleavage analysis and molecular cloning of partial digests were used to map the position of the Tn*1* insertions and one deletion on the pJM1 plasmid, which were manifested as either iron uptake–proficient or –deficient phenotypes (176).

Hence, a physical map of the location of the iron-uptake region on the pJM1 plasmid was obtained. The iron-uptake genes in pJM1 are located within a stretch of about 20–25 kb, which includes, possibly, the entire *BamHI* fragment 1 and parts of *BamHI* fragments 5 and 6 (176). Analysis of the outer membrane proteins of the iron uptake–deficient mutants that were able to be crossfed, for instance strains harboring plasmids like pJHC91 or pJHC-W7 (see Fig. 1), indicated that the Tn*1* insertions that affected the production of the putative siderophore neither affected receptor activity nor the biosynthesis of the OM2 protein.

In the case of the strain harboring the pJM1 deletion derivative pJHC9-8, both siderophore and receptor activity were affected. In this mutant the OM2 protein was no longer induced under conditions of iron limitation; instead, a novel protein of about 69 kd was synthesized. This protein could possibly be a truncated OM2 protein generated by Tn*1*-mediated deletion of pJM1 DNA or a fusion product. These results support the hypothesis that OM2 could be the plasmid-mediated receptor.

By using immunological and iodination experiments it was recently shown that the OM2 protein is actually exposed on the outside of the outer membrane layer, a result consistent with its possible role as a receptor (Actis, L., Potter, S. A. & Crosa, J. H., submitted for publication). The plasmid-mediated siderophore has not yet been identified.

Further characterization of the *V. anguillarum* plasmid-mediated iron-uptake system required the molecular cloning of specific pJM1 regions. Since the pJM1 plasmid–mediated system does not express in *E. coli* (Hodges & Crosa, unpublished observations), it was necessary to return the cloned regions to the *V. anguillarum* cytoplasm in order to study their expression. Most *E. coli* cloning vectors cannot be transferred to *V. anguillarum*. One exception is the broad host range R-plasmid RP4 that can be conjugated from *E. coli* to *V. anguillarum* (34). Consequently, a cloning vector derived from RP4 was the logical choice to initiate the genetic dissection of the pJM1 iron-uptake system.

The plasmid pVK102 is a small nonselftransmissible cosmid cloning vector constructed by Knauff & Nester (87) by ligating drug resistance genes and lambda cohesive ends into the plasmid pRK290 originally constructed by Ditta et al (38) from the R-plasmid RK2, which is identical to RP4. The pVK102 cosmid vector can be transferred from *E. coli* to *V. anguillarum* (Tolmasky, M. & Crosa, J. H., submitted for publication) by mobilization with the transfer factor pRK2013, also described by Ditta et al (38).

Clones of restriction endonuclease partial digests in pVK102 were transduced to *E. coli* after in vitro packaging and then conjugated to suitable *V. anguillarum* recipients by using three factor crosses with *E. coli* carrying pRK2013. Several cosmids containing DNA inserts with part or all of the pJM1 iron-uptake region, as mapped by Tn*1* insertion mutagenesis, were transferred

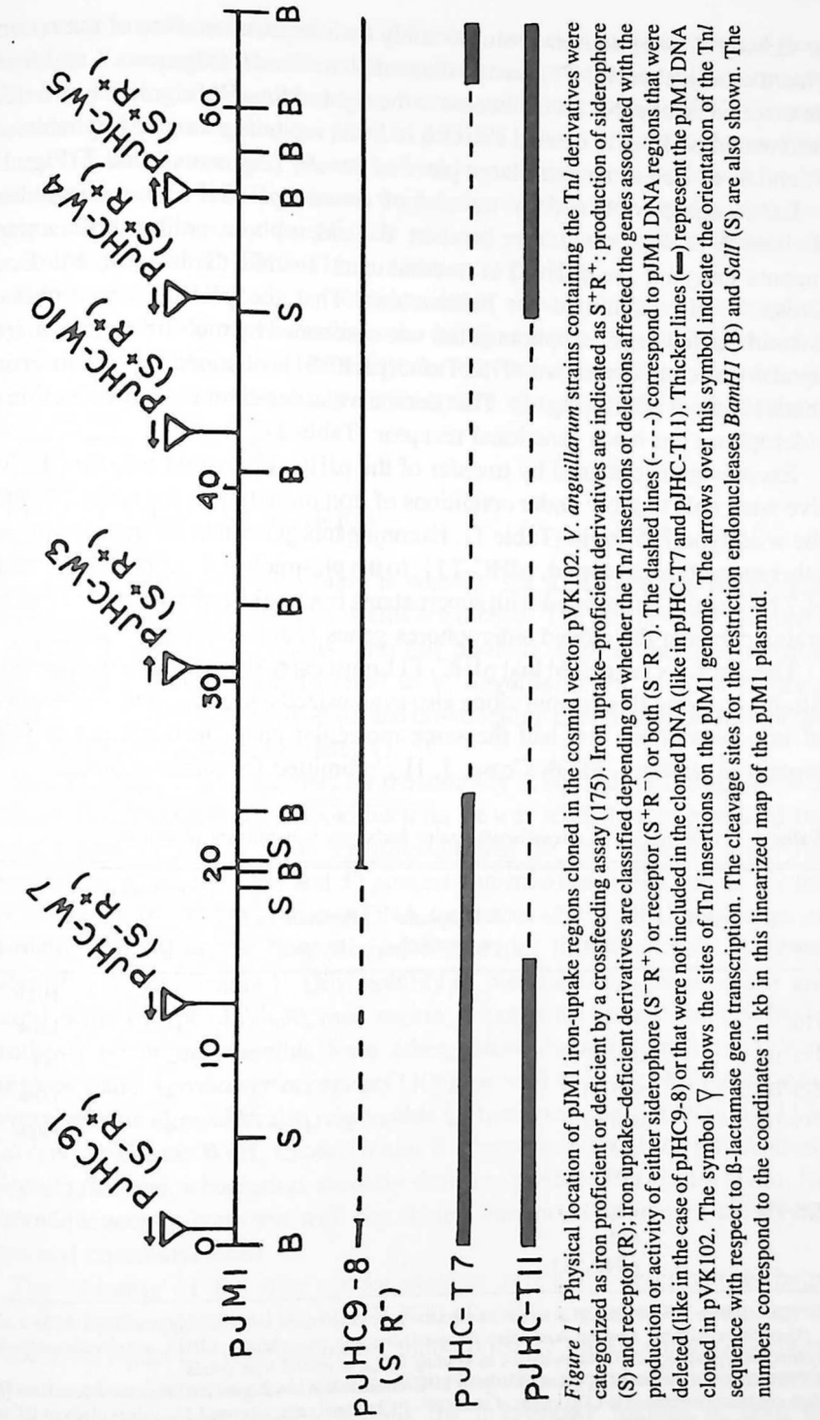


Figure 1 Physical location of pJM1 iron-uptake regions cloned in the cosmid vector pVK102. *V. anguillarum* strains containing the Tn*1* derivatives were categorized as iron proficient or deficient by a crossfeeding assay (175). Iron uptake–proficient derivatives are indicated as S^+R^+: production of siderophore (S) and receptor (R); iron uptake–deficient derivatives are classified depending on whether the Tn*1* insertions or deletions affected the genes associated with the production or activity of either siderophore (S^-R^+) or receptor (S^+R^-) or both (S^-R^-). The dashed lines (- - - -) correspond to pJM1 DNA regions that were deleted (like in the case of pJHC9-8) or that were not included in the cloned DNA (like in pJHC-T7 and pJHC-T11). Thicker lines (▬) represent the pJM1 DNA cloned in pVK102. The symbol ▽ shows the sites of Tn*1* insertions on the pJM1 genome. The arrows over this symbol indicate the orientation of the Tn*1* sequence with respect to β-lactamase gene transcription. The cleavage sites for the restriction endonucleases *BamH1* (B) and *Sall* (S) are also shown. The numbers correspond to the coordinates in kb in this linearized map of the pJM1 plasmid.

to various *V. anguillarum* strains to study their expression. One of the recombinant cosmids, pJHC-T7, carried the complete *BamHI* fragments 1 and 6 and an extra piece of pJM1 DNA located to the right of *BamHI* fragment 6, whereas the cosmid pJHC-T11 carried a stretch of DNA including *BamHI* fragments, 5, 7, and 8, as well as an extra-large piece of *BamHI* fragments 1 and 2 (Fig. 1).

Exconjugants obtained by transfer of cosmid pJHC-T7 to a plasmidless derivative of *V. anguillarum* produce the siderophore unlike those exconjugants carrying the pJHC-T11 recombinant cosmid (Tolmasky, M. E. & Crosa, J. H., submitted for publication). That the pJHC-T7 recombinant cosmid carried the siderophore genes was confirmed by mobilization to an iron uptake–deficient derivative, 775::Tn*1*-5(pJHC 91), obtained by Tn*1* insertion inactivation of pJM1 (Fig. 1). This derivative is deficient in the production of siderophore but has a functional receptor (Table 1).

Exconjugants obtained by transfer of the pJHC-T7 cosmid into this derivative were able to grow under conditions of iron limitation to the same extent as the wild-type 775 strain (Table 1). Exconjugants generated by transferring the other recombinant cosmid, pJHC-T11, to the plasmidless *V. anguillarum* strain H-775-3 could be crossfed with supernatants from wild-type strains or from the srains carrying the cloned siderophores genes (Table 1).

These results suggested that pJHC-T11 must carry the genes for the receptor. Strains carrying this cosmid clone also synthesized a protein, under conditions of iron restriction, that had the same molecular characteristics as the OM2 protein (Tolmasky, M. & Crosa, J. H., submitted for publication).

Table 1 Properties of *V. anguillarum* strains harboring recombinant plasmids

V. anguillarum strain	Plasmid	Siderophore production[a]	Presence of receptor[b]	Growth in 10 μM EDDA[c]	Virulence[d]
775	pJM1	+	+	+	High
775-3	–	–	–	–	Low
775-3C	pJHC-T11	–	+	–	NT
775-Tn*1*::5	pJHC91	–	+	–	Low
775-Tn*1*::5A	pJHC91 and pJHC-T7	+	+	+	High
775-Tn*1*::5C	pJHC91 and pJHC-T11	–	+	–	Low

[a,b]Siderophore production and presence of receptor were determined as previously described (175).

[c]Growth in minimal medium containing the nonassimilable iron chelator EDDA (ethylenediamine-di(o-hydroxyphenyl acetic acid) was measured by reading OD_{590} at several time points.

[d]Virulence was measured by determination of LD_{50} values (mean lethal doses) as previously described (34). High virulence correspond to LD_{50} values of about 10^2–10^3 bacterial cells, whereas LD_{50} values of about 10^8 cells are classified as low virulence. NT, not tested.

Another important result, shown in Table 1, is that transfer of some of these clones to *V. anguillarum* strains affects bacterial pathogenicity. Exconjugant 775::Tn*1*-5A harbors two plasmids, the recombinant cosmid pJHC-T7, with the siderophore genes, and the plasmid pJHC91, which possesses the receptor genes. This strain not only grows well under conditions of iron limitation but has also acquired full virulence, unlike the original iron uptake–deficient recipient harboring only the pJHC91 plasmid or exconjugants harboring the recombinant plasmid pJHC-T11 in addition to pJHC91.

Restoration of virulence by introduction of cloned pJM1 iron siderophore regions into a low-virulence strain is the fulfillment of a molecular approach to the Koch's postulates and demonstrates the intimate relation between the presence of the plasmid-mediated iron-uptake system and the high-virulence phenotype of *V. anguillarum*.

CONCLUDING REMARKS

One question that comes to mind is whether the pJM1 and the pColV-K30 plasmid-mediated iron-uptake systems are related. The pColV-K30 plasmid is conjugative, and DNA regions from this plasmid, such as the iron-transport genes, could have been transferred to *V. anguillarum* or to other bacteria. Nevertheless, recent hybridization and crossfeeding experiments indicated that these two systems were unrelated (175).

However, there is a precedent for the mobility of both pJM1 and pColV-K30 sequences between genomes and bacteria. It was recently demonstrated that certain virulent strains of *V. anguillarum* that do not posses a pJM1-like plasmid are iron-proficient and do possess chromosomal DNA sequences that are homologous to pJM1 plasmid DNA sequences (166). pJM1 sequences are also found in several *V. anguillarum* R-plasmids (Mitoma, Aoki & Crosa, submitted for publication). This mobility of plasmid DNA sequences is also found with the pColV-K30 iron region because the genes for aerobactin synthesis occur on plasmids from other enteric bacteria, such as *Arizona hinshawii* and *Aerobacter aerogenes* (106), as well as in the chromosomes of several enteric clinical strains responsible for human neonatal infections (112a; Valvano & Crosa; Wolf, Crosa, Woloj & Crosa, submitted for publication). *Shigella flexneri*, a bacterium recently shown to produce aerobactin (126), has also sequences in common with the cloned aerobactin sequences (S. Payne, personal communicaton).

The ubiquity of the iron uptake systems originally described as being encoded by the pJM1 and the pColV-K30 plasmids suggests that they may be respectively part of recombinational units, possibly transposition sequences, capable of epidemiological spread. Stressing this hypothesis is our recent finding that repeated sequences flank the iron-uptake regions of both the

pColV-K30 and the pJM1 plasmids (112A). Recombinational mobility may thus be an important common feature in the molecular evolution of these bacterial virulence determinants.

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Literature Cited

1. Andrus, C. R., Walter, M., Crosa, J. H., Payne, S. M. 1983. *Curr. Microbiol.* 9:209–14
2. Archibald, F. S., De Voe, I. W. 1979. *FEMS Microbiol. Lett.* 6:159–62
3. Beesley, E. D., Brubaker, R. R., Janssen, W. A., Surgalla, M. J. 1967. *J. Bacteriol.* 94:19–26
4. Bennett, R. L., Rothfield, L. I. 1976. *J. Bacteriol.* 127:498–504
5. Berger, H., Hacker, J., Juarez, A., Hughes, C., Goebel, W. 1982. *J. Bacteriol.* 152:1241–47
6. Bindereiff, A., Neilands, J. B. 1983. *J. Bacteriol.* 153:1111–13
7. Bindereiff, A., Braun, V., Hantke, K. 1982. *J. Bacteriol.* 150:1472–75
8. Binns, M. M., Davies, D. L., Hardy, K. G. 1979. *Nature* 279:778–81
9. Bjorn, J. J., Iglewski, B. W., Ives, S. K., Sadoff, J. C., Vasil, M. L. 1978. *Infect. Immun.* 19:785–91
10. Bjorn, J. J., Sokol, P. A., Iglewski, B. H. 1979. *J. Bacteriol.* 138:193–200
11. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., et al. 1977. *Gene* 2:95–113
12. Braude, A. I., Siemienski, J. S. 1965. *J. Clin. Invest.* 47:1763–73
13. Braun, V. 1981. *FEMS Microbiol. Lett.* 11:225–28
14. Braun, V., Burkhardt, R. 1982. *J. Bacteriol.* 152:223–31
15. Braun, V., Burkhardt, R., Schneider, R., Zimmermann, L. 1982. *J. Bacteriol.* 151:553–59

15a. Braun, V., Gross, R., Köster, W., Zimmermann, L. 1983. *Mol. Gen. Genet.* 192:131–39

16. Braun, V. Hancock, R. E. W., Hantke, K., Hartmann, A. 1976. *J. Supramol. Struct.* 5:37–58
17. Brubaker, R. R. 1970. *J. Bacteriol.* 98:1404–06
18. Brubaker, R. R., Beesley, E. D., Surgalla, M. J. 1965. *Science* 149:22–24
19. Buck M., Griffiths, E. 1981. *Nucleic Acids Res.* 9:401–14
20. Buck M., Griffiths, E. 1982. *Nucleic Acids Res.* 10:2609–24
21. Bullen, J. J. 1981. *Rev. of Infect. Dis.* 3:1127–38
22. Bullen, J. J., Armstrong, J. A. 1979. *Immunology* 36:781–91
23. Bullen, J. J., Leigh, L. C., Rogers, H. J. 1968. *Immunology* 15:581–88
24. Bullen, J. J., Rogers, H. J., Griffiths, E. 1978. *Curr. Top. Microbiol. Immunol.* 80:1–35
25. Canestrini, G. 1893. *Atti 1st Veneto Sci.* 7:809–14
26. Cisar, J. O., Fryer, J. L. 1969. *Bull. Wildl. Dis. Assoc.* 5:73–76
27. Clancy, J., Savage, D. C. 1981. *J. Bacteriol.* 32:343–52
28. Cox, C. D. 1982. *Infect. Immun.* 36:17–23
29. Cox, C. D., Rinehart, K. L. Jr., Moore, M. L., Carter Cook, J. Jr. 1981. *Proc. Natl. Acad. Sci. USA* 78:4256–60
30. Cox, G. B., Gibson, F., Luke, R. K. J., Newton, N. A., O'Brien, I. G., Rosenberg, H. 1970. *J. Bacteriol.* 142:581–87
31. Crosa, J. H. 1979. *Fish Health News* 8:7–9
32. Crosa, J. H. 1980. *Nature* 284:566–68
33. Crosa, J. H., Hodges, L. L. 1981. *Infect. Immun.* 31:223–27
34. Crosa, J. H., Hodges, L. L., Schiewe, M. H. 1980. *Infect. Immun.* 27:897–902
35. Crosa, J. H., Schiewe, M. H., Falkow, S. 1977. *Infect. Immun.* 18:509–13
36. Crosa, J. H., Walter, M. A., Potter, S. A. 1983. In *Microbiology—1983*, ed. D. Schlessinger, pp. 354–58. Washington DC: Am. Soc. Microbiol.
37. Crosa, J. H., Walter, M. A., Potter, S. A. 1983. In *Bacterial and Viral Diseases of Fish: Molecular Studies*, ed. J. H. Crosa, pp. 21–30. Seattle, Wash: Sea Grant
38. Ditta, G., Stanfield, S., Corbin, D., Helinski, D. R. 1980. *Proc. Natl. Acad. Sci. USA* 77:7347–51
39. Dubos, R. G., Geiger, J. 1946. *J. Exp. Med.* 84:143–56

40. Elwell, L. P., Shipley, P. L. 1980. *Ann. Rev. Microbiol.* 34:465–96
41. Ernest, J. F., Bennett, R. L., Rothfield, L. I. 1978. *J. Bacteriol.* 135:928–34
42. Evans, D. G., Silver, R. P., Evans, D. J., Chase, D. G., Gorbach, S. L. 1975. *Infect. Immun.* 12:656–67
43. Evelyn, T. P. T. 1971. *J. Fish. Res. Board Can.* 28:517–25
44. Ferber, D. M., Brubaker, R. R. 1981. *Infect. Immun.* 31:839–41
45. Ferber, D. M., Fowler, J. M., Brubaker, R. R. 1981. *J. Bacteriol.* 146:506–11
46. Finkelstein, R. A., Yancey, R. J. 1981. *Infect. Immun.* 32:609–13
47. Formal, S. B., Gemski, P. Jr., Baron, L. S., LaBrec, E. H. 1971. *Infect. Immun.* 3:73–79
48. Formal, S. B., LaBrec, E. H., Kent, T. H., Falkow, S. 1965. *J. Bacteriol.* 89: 1374–82
49. Frederick, P. 1951. *A. van Leeuwenhoek J. Microbiol. Serol.* 17:103–6
50. Frederick, P., Joiris, E. 1950. *C. R. Soc. Biol.* 144:435–37
51. Frost, G. F., Rosenberg, H. 1973. *Biochim. Biophys. Acta* 330:90–101
52. Gemski, P., Lazere, J. R., Casey, T. 1980. *Infect. Immun.* 27:682–85
53. Gemski, P., Sheahan, D. G., Washington, O., Formal, S. B. 1972. *Infect. Immun.* 6:104–11
54. Gibson, F., McGrath, D. I. 1969. *Biochim. Biophys. Acta* 6:104–11
55. Gratia, A. 1925. *C. R. Soc. Biol.* 93: 1040–41
56. Greenwood, K., Luke, R. K. J. 1980. *Biochim. Biophys. Acta* 614:185–95
57. Greenwood, K. T., Luke, R. K. J. 1978. *Biochim. Biophys. Acta* 525:209–18
58. Greenwood, K., Luke, R. K. J. 1981. *Biochim. Biophys. Acta* 660:371–74
59. Grewal, K. K., Warner, P. J., Williams, P. H. 1982. *FEBS Lett.* 140:27–30
60. Griffiths, E. 1983. In *Microbiology—1983*, ed. D. Schlessinger, pp. 329–33. Washington DC: Am. Soc. Microbiol.
61. Griffiths, E., Humphreys, J. 1978. *Eur. J. Biochem.* 82:503–13
62. Griffiths, E., Humphreys, J. 1980. *Infect. Immun.* 28:286–89
63. Griffiths, E., Humphreys, J., Leach, A., Scanlon, L. 1978. *Infect. Immun.* 22: 312–17
64. Griffiths, E., Stevenson, P., Joyce, P. 1982. *FEMS Microbiol. Lett.*
65. Griffiths, G. L., Sigel, S. P., Payne, S. M., Neilands, J. B. 1983. *J. Biol. Chem.* In press
66. Guterman, S. K. 1973. *J. Bacteriol.* 114:1217–24
67. Gyles, C. L., Palchaudhuri, S., Maas, W. K. 1977. *Science* 198:198–99
68. Gyles, C. L., So, M., Falkow, S. 1974. *J. Infect. Dis.* 130:40–49
69. Hale, T. L., Sansonetti, P. J., Schad, P. A., Austin, S., Formal, S. B. 1983. *Infect. Immun.* 40:340–50
70. Hancock, R. E. W., Hantke, K., Braun, V. 1976. *J. Bacteriol.* 127:1370–75
71. Hantke, K. 1981. *Mol. Gen. Genet.* 182:288–92
72. Hantke, K. 1982. *FEMS Microbiol. Lett.* 15:83–86
73. Hantke, K., Braun, V. 1975. *FEBS Lett.* 49:301–05
74. Hantke, K., Zimmermann, L. 1981. *FEMS Microbiol. Lett.* 12:31–35
75. Hedges, R. W., Jacob, A. E. 1974. *Mol. Gen. Genet.* 132:31–40
76. Hollifield, W. C., Neilands, J. B., 1978. *Biochemistry* 17:1922–28
77. Hoseith, S. K., Stocker, B. A. D. 1981. *Nature* 291:238–39
78. Horak, V., Lanc, A., 1973. *Folia Microbiol.* 18:332–40
79. Hussein, S., Hantke, K. Braun. V. 1981. *Eur. J. Biochem.* 117:431–37
80. Ichihara, S., Mizushima, S. 1977. *J. Biochem.* 81:749–56
81. Ichihara, S., Mizushima, S. 1978. *J. Biochem.* 83:137–40
82. Jackson, S., Burrows, T. W. 1956. *Br. J. Exp. Pathol.* 37:577–83
83. Kado, C. I. 1976. In *1st Beltsville Symp. Agriculture, Virology in Agriculture*, ed. J. A. Romberger, pp. 247–66. Allanheld, Osmun, Montclair, NJ
84. Kahn, P. L., Helinski, D. R. 1964. *J. Bacteriol.* 88:1573–79
85. Kaplan, S. S., Basford, R. E. 1979. In *Microbiology—1979*, ed. D. Schlessinger, pp. 92–95. Washington DC: Am. Soc. Microbiol.
86. Klebba, P. E., McIntosh, M. A., Neilands, J. B. 1982. *J. Bacteriol.* 149:880–88
87. Knauff, V., Nester, E. W. 1982. *Plasmid* 8:45–54
88. Konisky, J. 1979. In *Bacterial Outer Membranes: Biogenesis and Functions*, ed. M. Inouye, pp. 319–59. New York: Wiley
89. Konisky, J., Soucek, S., Frick, K., Davies, J. K., Hammond, C. 1976. *J. Bacteriol.* 127:249–57
90. Konopka, K., Bindereif, A., Neilands, J. B. 1982. *Biochemistry* 21:6503–8
91. Kopecko, D. J., Washington, O., Formal, S. B. 1980. *Infect. Immun.* 29:207–14
92. Krone, W. J. A., Luirink, J., Koningstein, G., Oudega, B., de Graaf, F. K. 1983. *J. Bacteriol.* 156:945–48

93. Krone, W. J. A., Oudega, B., Stegehuis, F., de Graaf, F. K. 1983. *J. Bacteriol.* 153:716–21
94. Laird, A. J., Ribbons, D. W., Woodrow, G. C., Young, I. G. 1980. *Gene* 11:347–57
95. Laird, A. J., Young, I. G. 1980. *Gene* 11:359–66
96. Langman, L., Young, I. G., Frost, G. E., Rosenberg, H., Gibson, F. 1972. *J. Bacteriol.* 112:1142–49
97. Lankford, C. E. 1973. *Crit. Rev. Microbiol.* 2:273–31
98. Leong, J., Neilands, J. B. 1976. *J. Bacteriol.* 126:823–30
99. Leong, S. A., Neilands, J. B. 1981. *J. Bacteriol.* 147:482–91
100. Locke, A., Main, E. R. 1931. *J. Infect. Dis.* 48:419–35
101. Luckey, M., Pollack, J. R., Wayne, R., Ames, B. N., Neilands, J. B. 1972. *J. Bacteriol.* 111:731–38
102. Luckey, M., Wayne, R., Neilands, J. B. 1975. *Biochem. Biophys. Res. Commun.* 64:689–93
103. Luke, R. K. J., Gibson, I. 1971. *J. Bacteriol.* 131:331–39
104. MacFarren, A. C., Clowes, R. C. 1977. *J. Bacteriol.* 84:365–77
105. McCandliss, R. J., Herrmann, K. M. 1978. *Proc. Natl. Acad. Sci. USA* 75:4810–13
106. McDougall, S., Neilands, J. B. 1983. *Abstr. Ann. Meet. Am. Soc. Microbiol., New Orleans,* p. 208
107. McIntosh, M. A., Earhardt, C. F. 1976. *Biochem. Biophys. Res. Commun.* 70:315–22
108. McLennan, B. D., Buck, M., Humphreys, J., Griffiths, E. 1981. *Nucleic Acids Res.* 9:2629–40
109. Mickelsen, P. A., Sparling, P. F. 1981. *Infect. Immun.* 33:555–64
110. Mickelsen, P. A., Blackman, E., Sparling, P. F. 1982. *Infect. Immun.* 35:915–20
111. Mietzner, T., Morse, S. A. 1983. *Abstr. 5th Meet. Int. Soc. Sexual. Transm. Dis. Res., Seattle,* p. 100
112. Minshew, B. H., Jorgensen, G., Counts, G. W., Falkow, S. 1978. *Infect. Immun.* 20:50–54

112a. Mitoma, Y., Perez-Casal, J., Walter, M. A., Crosa, J. H. 1984. *Abstr. Ann. Meet. Am. Soc. Microbiol., St. Louis,* p. 46

113. Moll, A., Manning, P. A., Timmis, K. N. 1980. *Infect. Immun.* 28:359–67
114. Moore, D. G., Yancey, R. J., Lankford, C. E., Earhart. 1980. *Infect. Immun.* 27:418–23
115. Moore, D. G., Earhart, C. F. 1981. *Infect. Immun.* 31:631–35
116. Nagel de Zwaig, R., Anton, D. 1964. *Biochem. Biophys. Res. Commun.* 17: 358–62
117. Neilands, J. B. 1957. *Bacteriol. Rev.* 21:101–11
118. Neilands, J. B. 1981. *Ann. Rev. Biochem.* 50:715–31
119. Neilands, J. B. 1982. *Ann. Rev. Microbiol.* 36:285–309
120. Norqvist, A., Davies, J., Norlander, L., Normark, S. 1978. *FEMS Microbiol. Letts.* 4:71–75
121. Narrod, P., Williams, R. P. 1978. *Curr. Microbiol.* 1:281–84
122. O'Brien, E. G., Gibson, F. 1970. *Biochim. Biophys. Acta* 215:393–402
123. Oudega, B., Oldenziel-Werner, W. J. M., Klaasen-Boor, P., Rezee, A., Glas, J., de Graaf, F. K. 1979. *J. Bacteriol.* 138:7–16
124. Orskov, I., Orskov, F. 1966. *J. Bacteriol.* 91:69–75
125. Pappenheimer, A. M. Jr. 1977. *Ann. Rev. Biochem.* 46:69–94
126. Payne, S. M. 1980. *J. Bacteriol.* 143: 1420–24
127. Payne, S. M., Finkelstein, R. A. 1975. *Infect. Immun.* 12:1313–18
128. Payne, S. M., Finkelstein, R. A. 1978. *Infect. Immun.* 20:310–11
129. Payne, S. M., Finkelstein, R. A. 1978. *J. Clin. Invest.* 61:1428–40
130. Perry, R. D., Brubaker, R. R. 1979. *J. Bacteriol.* 137:1290–98
131. Pierce, J. R., Pickett, C. L., Earhardt, C. F. 1983. *J. Bacteriol.* 155:330–36
132. Plastow, G. S., Holland, I. B. 1979. *Biochem. Biophys. Res. Commun.* 90: 1007–14
133. Plastow, G. S., Pratt, J. M., Holland, I. B. 1981. *FEBS Lett.* 31:262–64
134. Pollack, J. R., Ames, B. N., Neilands, J. B. 1970. *J. Bacteriol.* 104:635–39
135. Pollack, J. R., Neilands, J. B. 1970. *Biochem. Biophys. Res. Commun.* 38: 989–92
136. Portnoy, D. A., Moseley, S. L., Falkow, S. 1981. *Infect. Immun.* 31:775–82
137. Portnoy, D. A., Falkow, S. 1981. *J. Bacteriol.* 148:877–83
138. Postle, K., Reznikoff, W. 1979. *J. Mol. Biol.* 131:619–36
139. Pugsley, A. P., Reeves, P. J. 1976. *J. Bacteriol.* 126:1052–62
140. Quackenbusch, R. L., Falkow, S. 1979. *Infect. Immun.* 24:562–64
141. Rogers, H. J. 1973. *Infect. Immun.* 7:445–56
142. Rosenberg, H., Young, I. G. 1974. In *Microbial Iron Metabolism,* ed. J. B. Neilands, pp. 67–82. New York: Academic
143. Rucker, R. R., Earp, B. J., Ordal, E. J. 1953. *Trans. Am. Fish. Soc.* 83:297–312

144. Russell, L. M., Holmes, R. K. 1983. *J. Bacteriol.* 155:1439–42
145. Sansonetti, P. J., David, M., Toucas, M. 1980. *C. R. Acad. Sci. Paris Ser. D* 290:879–82
146. Sansonetti, P. J., Kopecko, D. J., Formal, S. B. 1981. *Infect. Immun.* 34:75–83
147. Sansonetti, P. J., Kopecko, D. J., Formal, S. B. 1982. *Infect. Immun.* 35:52–60
148. Schneider, R., Hartmann, A., Braun, V. 1981. *FEMS Microbiol. Lett.* 11:115–19
149. Shade, A. L., Caroline, L. 1946. *Science* 104:340–41
150. Sigel, S. P., Payne, S. M. 1982. *J. Bacteriol.* 150:148–55
151. Simonson, C., Brener, D., De Voe, I. W. 1982. *Infect. Immun.* 36:107–13
152. Simpson, L. M., Oliver, J. D. 1983. *Infect. Immun.* 41:644–49
153. Smith, H. W. 1974. *J. Gen. Microbiol.* 83:95–111
154. Smith, H. W. 1976. In *Acute Diarrhoea in Childhood,* ed. K. Elliott, J. Knight, pp. 44–72. Amsterdam: Elsevier
155. Smith, H. W., Halls, S. 1967. *J. Gen. Microbiol.* 47:153–67
156. Smith, H. W., Halls, S. 1968. *J. Gen. Microbiol.* 52:319–34
157. Smith, H. W., Huggins, M. B. 1976. *J. Gen. Microbiol.* 92:335–50
158. Smith, H. W., Huggings, M. B. 1977. *J. Med. Microbiol.* 10:479–82
159. Smith, H. W., Linggood, M. A. 1972. *J. Med. Microbiol.* 5:243–50
160. Smith, H. W., Parsell, Z. 1975. *J. Gen. Microbiol.* 87:129–40
161. So, M., Crosa, J. H., Falkow, S. 1975. *J. Bacteriol.* 121:234–38
162. Straley, S. C., Brubaker, R. R. 1982. *Infect. Immun.* 36:129–35
163. Stuart, S. J., Greenwood, K. T., Luke, R. K. J. 1980. *J. Bacteriol.* 143:35–42
164. Stuart, S. J., Greenwood, K. T., Luke, R. K. J. 1982. *Infect. Immun.* 36:870–75
165. Taylor, P. W., Hughes, C. 1978. *Infect. Immun.* 22:10–17
166. Toranzo, A. E., Barja, J. L., Potter, S. A., Colwell, R. R., Hetrick, F. M., Crosa, J. H. 1983. *Infect. Immun.* 39:1220–27
167. Uemura, J., Mizushima, S. 1975. *Biochim. Biophys. Acta* 413:163–73
168. Van den Bosch, J. F., Postma, P., de Graaff, J., MacLaren, D. M. 1981. *J. Med. Microbiol.* 14:321–31
169. Van Heyningen, W. E., Gladstone, G. P. 1953. *Br. J. Exp. Pathol.* 34:221–29
170. Van Larebeke, N., Engler, G., Hosters, M., Van den Elsacker, S., Zaenen, I., et al. 1974. *Nature* 252:169–70
171. Van Tiel-Menkveld, G. J., Mentjox-Vervuurt, J. M., Oudega, B., de Graaf, F. K. 1982. *J. Bacteriol.* 150:490–97
172. Van Tiel-Menkveld, G. J., Oudega, B., Kempers, O., de Graaf, F. K. 1981. *FEMS Microbiol. Lett.* 12:373–80
173. Wagegg, W., Braun, V. 1981. *J. Bacteriol.* 145:156–63
174. Wake, A., Misawa, M., Matsui, A. 1975. *Infect. Immun.* 12:1211–13
175. Walter, M. A., Bindereif, A., Neilands, J. B., Crosa, J. H. 1984. *Infect. Immun.* 43:765–67
176. Walter, M. A., Potter, S. A., Crosa, J. H. 1983. *J. Bacteriol.* 156:880–87
177. Waring, W. S., Werkman, C. H. 1942. *Arch. Biochem.* 4:75–87
178. Warner, P. J., Williams, P. H., Bindereif, A., Neilands, J. B. 1981. *Infect. Immun.* 33:540–45
179. Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D., Nester, E. W. 1975. *J. Bacteriol.* 123:255–64
180. Wayne, R., Neilands, J. B. 1975. *J. Bacteriol.* 121:497–503
181. Weaver, C. A., Konisky, J. 1980. *J. Bacteriol.* 143:1513–18
182. Welch, R. A., Dellinger, E. P., Minshew, B., Falkow, S. 1981. *Nature* 294:665–67
183. Weinberg, E. D. 1974. *Science.* 184:952–56
184. Weinberg, E. D. 1978. *Microbiol. Rev.* 42:45–66
185. Wilkins, T. D., Lankford, C. E. 1970. *J. Infect. Dis.* 121:129–36
186. Williams, P. H. 1979. *Infect. Immun.* 26:925–32
187. Williams, P. H., George, H. K. 1979. In *Plasmids of Medical, Environmental and Commercial Importance.* Amsterdam: Elsevier-North Holland
188. Williams, P. H., Warner, P. J. 1980. *Infect. Immun.* 29:411–16
189. Woodrow, G. C., Langman, L., Young, I. G., Gibson, F. 1978. *J. Bacteriol.* 133:1524–26
190. Wookey, P. J., Hussein, S., Braun, V. 1981. *J. Bacteriol.* 146:1158–61
191. Wookey, P. J., Rosenberg, H. 1978. *J. Bacteriol.* 133:661–66
192. Wright, A. C., Simpson, L. M., Oliver, J. D. 1981. *Infect. Immun.* 34:503–7
193. Yancey, R. J., Breeding, S. A. L., Lankford, C. E. 1979. *Infect. Immun.* 24:174–80
194. Yancey, R. J., Finkelstein, R. A. 1981. *Infect Immun.* 32:592–99
195. Yancey, R. J., Finkelstein, R. A., 1981. *Infect. Immun.* 32:600–8
196. Young, I. G., Langman, L., Luke, R. K. J., Gibson, F. 1971. *J. Bacteriol.* 106: 51–57
197. Zink, D. L., Feeley, J. C., Wells, J. G., Vanderzant, C., Vickery, J. C., et al. 1980. *Nature* 283:224–26

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INHIBITION OF CELL FUNCTIONS BY RNA-VIRUS INFECTIONS

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INTRODUCTION

One typical consequence of a virus infection is cell destruction, which in the host may cause more or less permanent functional disturbances—even kill the host. Studies of virus-infected cell cultures revealed, early on, that a variety of viruses effectively inhibit host-cell macromolecular synthesis.

This review is limited to RNA viruses, which offer the simplest models due to their limited genetic capacity. The structure and mode of RNA virus replication have been solved to a large extent. Of these viruses, poliovirus—a member of the picornaviruses—has been best characterized. Vesicular stomatitis virus (VSV), a member of the rhabdoviruses, has served as another model. The work with alphaviruses and reoviruses is also reviewed, although they have been less extensively studied than poliovirus and VSV.

We have excluded other RNA viruses, such as myxo-, paramyxo-, and bunyaviruses, many of which also cause inhibition of host cell functions (12, 22, 24). Most of the recent work has concentrated on solving the replication processes of these viruses rather than their ability to inhibit host functions (84, 132).

STRUCTURE AND REPLICATION OF PICORNAVIRUSES

The poliovirion contains a single-stranded infectious RNA molecule. Its nucleotide sequence, gene order, and the mode of gene expression are known (80, 105). The viral RNA of 7410 nucleotides in length is translated into a large polyprotein, starting from an AUG codon 743 nucleotides from the 5' end of the genome and extending to a termination codon 71 nucleotides from the 3' end. This polyprotein precursor is processed by proteolytic cleavages to yield the final poliovirus proteins, which include the four viral structural proteins (VP1–VP4) (9, 68), the RNA polymerase (38), a virus-specific proteolytic enzyme (57), and the fifth virion associated protein, the VPg (150), which is covalently bound to the 5' end of both viral plus and minus strands. The functions of some of the gene products derived from the central part of the polyprotein, representing about a quarter of the genetic coding capacity, are unknown. The RNA is replicated in the cytoplasm by the virus coded RNA polymerase which most probably functions in association of a defined host component (for references see 27).

Viral mRNA is identical to the virion RNA except that the VPg protein has been cleaved off its 5' end. The mRNA has instead a free 5' phosphate (64), thereby differing from the bulk of cellular and other virus coded messengers, which all contain the cap-structure at their 5' end (for review see 10). The unusual lack of cap from poliovirus RNA is thought to allow distinction between viral and cellular mRNA during translation in an infected cell and thus provide an explanation for the translational control that takes place during poliovirus infection.

INHIBITION OF HOST PROTEIN SYNTHESIS BY PICORNAVIRUSES

Poliovirus infection in HeLa cells causes a dramatic change in the host cell functions early in the infectious cycle (92). Within three hours the total rate of protein synthesis has dropped to about 50%; it further decreases to around 20% towards the end of infection. Three hours after infection most of the proteins produced are virus-specific. The host shut-off is also manifested as an inhibition of cellular RNA and DNA synthesis.

In early studies with poliovirus (92) the conclusion was reached that the mechanism of inhibition of host-cell protein synthesis is dependent on poliovirus-directed protein synthesis and takes place at the initiation step of translation (7, 32, 53). The evidence supporting this conclusion as well as the alternative hypotheses, which have been shown not to be valid, are briefly reviewed below.

If cells are infected with UV-irradiated virus, no virus-specific translation is possible. Under such conditions host functions are not shut off (61). This indicates that the incoming viral components are not sufficient to bring about the inhibition of the host.

Host-cell protein synthesis is inhibited under conditions where poliovirus RNA synthesis is prevented, but primary translation occurs. This is achieved by infecting cells at a high multiplicity of infection in the presence of guanidine, which selectively inhibits viral RNA synthesis but does not interfere with translation of parental viral RNA (65).

Those viral gene products required to turn off host functions—if any—are not clear. Some ts-mutants of poliovirus, defective in the production of structural proteins, were unable to shut off the host at the nonpermissive temperature (130). On the other hand, infection with a defective interfering particle, which had a deletion in the structural protein genes, was able to effectively cause inhibition of host protein synthesis (23)

Excluded Mechanisms

INSTABILITY OF HOST mRNA During picornavirus infections the amount and size of host mRNA remain roughly unchanged (92) as demonstrated with the histone messenger in Ehrlich ascites cells infected with mengovirus. Quantitation of the mRNA by in vitro translation revealed that 50% of the histone mRNA was present in the cells 4 hours after infection, whereas the level of histone synthesis had decreased to 10% of that before infection (41). Fernan-

dez-Munoz & Darnell (36) examined the 5' ends of prelabeled polyA-containing mRNA from HeLa cells 2 hours after poliovirus infection, from mock infected cells and cells treated with puromycin. No changes were detected in the mRNA populations.

CELL PERMEABILITY CHANGES Altered permeability of cellular membranes and thereby increased concentration of monovalent cations inside the cells was postulated as a possible mechanism for specific inhibition of host protein synthesis (19). Such changes apparently occur as a result of infection, and they have an effect on translation as measured in vitro. The changes, however, are manifested too late to explain the shut-off. Furthermore, it was recently shown (85) that the modification in the permeability of HeLa cells to ^{86}Rb ions, induced by poliovirus, was prevented by guanidine, which in itself does not interfere with the host shut-off. Changes in cell membrane permeability have not been observed during SFV and VSV infections (54).

DOUBLE-STRANDED RNA Inhibition of protein synthesis by dsRNA has been ruled out as the mechanism of host shut-off by poliovirus. The original observation showed that an extract from poliovirus-infected cells inhibited protein synthesis in vitro in a reticulocyte lysate (67), and the inhibitory activity was assigned to poliovirus dsRNA in the extract (31). However, dsRNA was equally potent in inhibiting cellular as well as poliovirus RNA translation (20). Lack of specificity is viewed as evidence that dsRNA is not involved in turning off the host in poliovirus infected cells (17).

Initiation Factors

Efficient in vitro translation systems have been successfully applied to studying the role of initiation factors in translating both poliovirus RNA and various capped mRNAs. Helentjaris & Ehrenfeld (62) demonstrated that initiation factors isolated from poliovirus-infected cells were unable to promote initiation of translation on host polysomes, but were active on poliovirus polysomes. A comparable preparation of crude ribosomal salt wash from uninfected cells was capable of supporting the initiation on host mRNA and poliovirus RNA. This suggested that the initiation factors during poliovirus infection had selectively lost the ability to recognize capped mRNA. This finding has been confirmed by more refined cell-free extracts dependent on exogeneous mRNA (17, 18).

In EMC-virus-infected cells, inhibition of host-cell protein synthesis is less dramatic than in cells infected with poliovirus. The capacity to translate capped mRNAs was not lost from extracts prepared from these cells (71). This apparent discrepancy between the two picornaviruses might be due to the difference in the 5' end structure of these RNAs (35, 102).

Using a slightly different approach, Rose et al (110) concluded that a particular initiation factor fraction, which recognizes the mRNA, was inactivated during poliovirus infection. In a cell free extract, prepared from poliovirus-infected HeLa cells and treated with micrococcal nuclease, poliovirus RNA was translated whereas capped VSV mRNA was not. Translation of VSV mRNAs could be restored by the addition of an initiation factor preparation from salt-washed rabbit reticulocyte ribosomes. The restoring activity copurified with the eIF-4B. An analogous assay, studying the activity of various factors to restore translation of capped mRNAs in a deficient cell extract, has been widely used to identify components lacking from poliovirus-infected cells (e.g. 63).

Cap-Binding Protein (CBP)

The activity restoring the translation of capped mRNAs in poliovirus-infected cell extracts has been purified and found to be associated with the cap-binding protein complex (29, 126, 127, 134, 136).

Using UV cross-linking (124), Sonenberg et al (125) showed that a 24K protein present in initiation factor preparations (e.g. eIF-3 or eIF-4B) was cross-linked to the 5' cap of reovirus mRNA. This protein, when purified to apparent homogeneity, was shown to stimulate translation in vitro of capped messengers (126).

The restoring activity, purified from the eIF-3 fraction, when cross-linked to cap showed the presence of the 24K CBP, but the restoring activity of the purified CBP was labile (136). Translation of capped mRNA was inhibited by addition of monoclonal antibody directed against the 24K CBP. The degree of inhibition varied depending on the secondary structure of the 5' end of the RNA studied, but the translation of uncapped mRNA was unaffected (128). The antibody also interfered with the initiation complex formation on reovirus mRNA.

Further analysis of the CBP revealed that the functional form is probably a high-molecular-weight complex composed of the 24K CBP together with several other components as large as 220K (29, 55, 123, 133, 134). Using cross-linking to the reovirus mRNA cap as measure, cap-binding was shown to be a complex process: In the absence of ATP only the 24K protein binds, whereas in the presence of ATP several other components are also bound, among them eIF-4A and eIF-4B (29, 123).

Evidently, the recognition of cap occurs via the 24K CBP followed by an ATP-dependent step, possibly unfolding of the secondary structure of the mRNA 5' end and binding of subsequent initiation factors (29, 128). The 24K protein is antigenically related to some of the larger proteins, suggesting that it may be derived from a larger precursor by proteolytic processing (34).

DISSOCIATION OF CBP FROM eIF-3 Lee & Sonenberg (87) showed that the quantity of the CBP, as analyzed by the cross-linking assay, was diminished in poliovirus-infected cells. The CBP was present in the ribosomal salt wash but was apparently inactive, because only preparations from uninfected HeLa cells could restore the translation of capped mRNA (58). These partially contradictory results were explained by Hansen et al (59), who showed that the CBP from uninfected cells cosedimented with eIF-3, whereas in polio-infected cells the CBP was exclusively associated with slowly sedimenting fractions. Thus, poliovirus infection leads to the dissociation of CBP from the initiation factor complex.

Experiments trying to demonstrate inhibition of translation of capped mRNAs in vitro by extracts from poliovirus-infected cells have been unsuccessful (87, 110). An inhibitory effect manifested only after preincubation of the initiation factor preparations both from the infected and uninfected cells (87). Thus, it remains unclear whether poliovirus itself specifies the dissociation of CBP from the initiation factors (eIF-3).

Conclusions

Several groups have demonstrated that initiation factors isolated from poliovirus-infected cells are unable to support initiation of translation of capped mRNA. This inability is connected with the inactivation of a cap-binding activity, which probably is due to dissociation of the cap-binding protein(s) (CBP) from a large initiation factor complex, eIF-3 (30). Whether the dissociation is caused by a poliovirus gene product has not been determined.

By the time host protein synthesis is shut off the proportion of viral mRNA has increased considerably; the number of available ribosomes most probably becomes limiting in initiation complex formation. Thus, inhibition of host mRNA translation might simply be a result of competition. If so, the question of how the competition explains the inactivation of the CBP remains unanswered. Because the CBP is not needed in the initiation complex formation with poliovirus RNA, it is tempting to assume that the CBP becomes excluded from the active initiation factors and consequently depleted from the bulk of the initiation factors as uncapped mRNA is continuously translated. This would mean that there is a continuous association-dissociation of the CBP with, say, eIF-3 in normal cells when capped mRNAs are translated. In poliovirus-infected cells the discriminated CBP loses its function and its ability to reassociate with the eIF-3.

The assumption that inhibition of host protein synthesis is due to competition between host and virus mRNA is supported by double infection experiments with a picornavirus and a virus that produces capped mRNA. The amount of translation of capped viral mRNA as compared to poliovirus RNA is dependent on the ratios of the respective mRNAs (2, 3, 101).

INHIBITION OF HOST RNA AND DNA SYNTHESIS BY PICORNAVIRUSES

Following picornavirus infection of mammalian cells, host-cell RNA synthesis is rapidly inhibited to less than 10% of the initial level (92). This is true for polio-, mengo-, and EMC-virus. RNA polymerase I activity responsible for ribosomal RNA synthesis as well as RNA polymerase II transcribing mRNA were both shown to be inhibited (4, 25, 114). The RNA polymerases, when solubilized from infected cells, were however, active when assayed under conditions where only nonspecific initiation occurs (4, 5, 39, 114). Cellular DNA synthesis is also inhibited during picornavirus infection, but later and less dramatically than the RNA synthesis (56). The mechanism of this inhibition has not been studied recently.

Cell-free transcription systems have enabled a new approach to study the mechanism by which poliovirus turns off cellular RNA polymerases. Using in vitro transcription, Crawford et al (26) analyzed the RNA polymerase II and III activities in poliovirus-infected and uninfected cell extracts by addition of defined DNA fragments, one containing the major adenovirus type 2 late promoter and the other the adenovirus type 2 associated genes (VA). The former DNA is transcribed by RNA polymerase II and the latter by RNA polymerase III.

Preparing the extracts at hourly intervals, it was shown that polymerase II activity was inhibited to almost zero by 3 hours after infection, whereas polymerase III was only inhibited to 30% of the initial value by 4 hours after infection. The decrease of RNA polymerase II activity was parallel to the inhibition of cellular RNA synthesis, as measured in vivo. The RNA polymerase II–dependent transcription in the poliovirus-infected cell extract could be restored by addition of a transcription factor present in the S100 from uninfected cells. Purified polymerase II did not restore the activity. This implies that at least one factor required for specific transcription by polymerase II is deficient in extracts from poliovirus-infected cells. Thus, the inhibition is not due to inactivation of the polymerase II alone.

INHIBITION OF HOST FUNCTIONS BY ALPHAVIRUSES

The models for alphaviruses have been mostly Semliki Forest virus (SFV) and Sindbis virus (SIN). These viruses have a capped and polyadenylated single-stranded RNA with positive polarity of about 12,000 nucleotides. Capsid protein (264–266 amino acids) is intimately associated with the RNA in the nucleocapsid, which is surrounded by a lipoprotein membrane. Two glycoproteins, E1 (438–439 amino acids) and E2 (422–423 amino acids), are anchored to the lipids by their carboxy-terminal hydrophobic segment. In SFV there is a third peripheral glycoprotein, E3 (66 amino acids) (42, 75, 131, 132).

A subgenomic mRNA, 26S RNA (about 4100 nucleotides) serves as messenger for the structural proteins, which are translated as a polyprotein. The capsid protein binds to the polysomal ribosomes during the translation (49, 122, 138, 139) and is released to the nucleocapsid either directly from the polysomes or from the monosome pool (139). The envelope glycoproteins are transported to the plasma membrane (42), where they recognize the nucleocapsid during virus maturation. Four nonstructural proteins are translated from the 42S RNA also as a polyprotein (75, 79, 132). In SFV infected cells one of the nonstructural proteins is found in association with ribosomes, together with the capsid-protein (106).

Inhibition of Host Protein Synthesis

In the middle of infection the host protein synthesis is shut off fairly efficiently (60, 77, 93, 146), and mostly structural proteins of the virus are translated (86). Quantitation of the viral and cellular mRNAs using ^{32}P-equilibrium labeling (137) revealed that there are about equal amounts of viral and host mRNAs at 4 hours after infection. It was suggested that host protein synthesis was shut off by competition (75, 137, 146, 147).

Garry et al (43) suggested that the inhibition of host translation is due to a decreased K^+ and an increased Na^+ concentration in the infected cells, due to the effect of viral infection to the Na^+/K^+ pump (44). Gray et al (54) confirm that there is a slight increase in the intracellular concentration of Na^+, but no decrease in K^+ ion concentration. Whether this is connected with the host protein synthesis inhibition remains unclear.

In the ribosomal wash fraction of SFV-infected neuroblastoma cells, van Steeg et al (143) observed reduced amounts of initiation factors capable of translating capped mRNAs of host and early SFV RNAs. EMC-virus mRNA and late SFV RNAs could still be translated. The defect could be restored by purified eIF-4B and the CBP from reticulocytes.

These results could be explained by assuming that late in SFV infection the translation of structural proteins would take place from 42S RNA by internal initiation (142). Translation of structural proteins from 42S RNA has been reported (50, 121, 142), but it has not been possible to verify those results with intact 42S RNA preparations (48, 51). Atkins (6) studied temperature-sensitive mutants of SFV and came to the conclusion that structural proteins are involved in the shut-off of host protein synthesis.

Studies with SFV mutants have clearly given a correlation between the shut-off of cell protein synthesis and the amount of viral RNA. RNA-negative mutants, which apparently display only the primary translation, do not inhibit host protein synthesis (78). Mutants with reduced RNA synthesis ability ($RNA^{\pm}$) (10–50%) show concomitant translation of host and viral proteins late in infection (76, 77). These results fit well with the competition model by

Lodish & Porter (89, 90) for VSV, for which no special advantage in protein synthesis initiation is needed for the viral mRNA (146).

Inhibition of Host DNA Synthesis

The inhibition of host DNA synthesis has been studied using western equine encephalitis virus (WEE) as a model in BHK21 cells (81, 82, 117). Infected cell extracts contained a heat labile factor that inhibited the activity of DNA polymerase in vitro. Addition of dNTPs competed with the inhibitory activity efficiently. There was an enzyme that copurified with the inhibitory activity, a nucleotide triphosphate phosphohydrolase. Whether this is WEE virus-coded or induced, is not known.

Conclusions

For host-cell protein synthesis inhibition, three of several mechanisms that have been suggested are: (*a*) An imbalance of K^+/Na^+ ratio, which affects initiation; more experimental work is required to test this hypothesis. (*b*) A specific decrease in the activity that translates capped mRNAs, but late in infection capped 26S mRNA is found in the polysomes synthesizing exclusively and efficiently structural proteins (75). (*c*) A competition model has been postulated that does not require specificity from the viral mRNAs.

If the viral products have any specific effect on the translational machinery, it would be tempting to suggest that the specific binding of capsid protein to the ribosomes would be involved in this regulation (138, 139).

STRUCTURE AND REPLICATION OF VSV

Vesicular stomatitis virus is an enveloped, bullet-shaped RNA virus. The surface spikes are composed of a single glycoprotein species, G-protein (511 amino acids) (109), which are anchored to the lipid bilayer. On the inside of the bilayer there is the M-protein (229 amino acids) (109), which mediates the contact between G-protein and the nucleocapsid. The helical RNP consists of about 2000 copies of N-protein (422 amino acids), NS-protein (222 amino acids) (40) and L-protein (about 160 Kd) in 200 and 50 copies per virion, respectively, and one 42 S RNA molecule with negative polarity (about 11,000 nucleotides). The genome codes for five different proteins—all of which are found in the virion—in the following order: 3'N-NS-M-G-L-5' (1, 8). L- and NS-proteins are responsible for the virion transcriptase activity.

After entering the host cell, presumably by adsorptive endocytosis (97), the virion transcriptase is activated, leading to *primary transcription* of five capped and methylated, polyadenylated mRNAs as deduced by in vitro experiments (for review see 132). The transcription starts from a single promoter at the 3' end, resulting in the synthesis of a leader RNA of 47 nucleotides (13, 14, 88)

apparently followed by termination and reinitiation at the promoter of the N-gene. At the end of this gene there is a common signal, 3'AUACU$_7$5', for polyadenylation (108).

After addition of poly(A) the transcription of the NS gene starts, followed by M-, G-, and L-genes by a similar mechanism. This primary transcription represents about 10% of all virus-specific mRNA and is insensitive to protein synthesis inhibitors (132, 140). One product of the primary translation is N-protein, which accumulates and evidently binds to the plus-leader RNA during transcription, preventing the premature termination and re-initiation. This leads to the synthesis of full-length plus-antigenome, which is also packed into RNP (13, 14, 112). The antigenomes are then replicated into new minus strands (RNPs), which start the *secondary transcription*, translation and replication thus amplifying the virus infection. The function of the leader sequence in minus and plus strand RNA is to serve as an encapsidation signal, which is not present in the viral mRNAs.

As a result of the secondary translation large amounts of virus-specific proteins are synthesized. G-protein is transported to the plasma membrane through the Golgi complex. N, NS, and L proteins associate with either plus or minus strands as RNPs and M-protein recognizes the G-protein at the plasma membrane and serves as a target for RNPs during the maturation of virions (74, 132, 140). A typical feature in VSV infection is the rapid shut-off of host macromolecular synthesis that takes place 3–4 hours after infection (140).

INHIBITION OF HOST PROTEIN SYNTHESIS BY VSV

Competition Model

Lodish & Porter (89) studied wild type VSV infected cells in suspension culture 2.5–4.5 hours after infection. In their careful study they determined the amount of virus-specific proteins and chose 10 major host proteins for comparison. The overall protein synthesis activity in the infected cells was slightly lower than in the mock-infected cells (e.g. 75% at 3.5 hr after infection). In vitro translation of polysomal RNAs revealed that actin mRNA was in fractions containing 12 ribosomes.

In VSV-infected cells the amount of total translatable mRNA increased during the course of infection, in spite of the overall reduction of the protein synthesis activity. There was a clear shift of mRNAs to smaller polysomes, and actin mRNA became associated with pentasomes. The host mRNAs were neither inactivated nor specifically excluded from the polysomes.

Lodish & Porter postulated that the host-cell inhibition is simply due to competition for a limiting amount of ribosomes. The new, virus-specific mRNA, which soon gains an excess, has the same probability to initiate protein synthesis as the host mRNA. This leads to a competition at the level of initiation

resulting in polysomes with fewer ribosomes for both viral and host mRNA. At 4.5 hr after infection the ratio of viral to cellular mRNA was 2–3.5:1, which fully explains the observed 2–3-fold reduction in the synthesis of the major cellular proteins.

In their second paper (90) the authors used three additional VSV-strains and two different cell lines (Vero and mouse L-cells), which were grown in monolayer cultures. The different virus-cell combinations were analyzed as before. There was a clearcut correlation between the amount of viral mRNA and the degree of host-cell protein synthesis inhibition. In L-cells, for example, the rate of shut-off was slower than in BHK21 cells, correlating with a slower rate of accumulation of viral mRNA.

This model as applied to VSV wild-type infected cells in the middle of the growth cycle explains the host-cell protein synthesis inhibition without any specific roles of viral RNAs or proteins on the translational machinery. However, this model has been challenged by two groups. Jaye et al (69) observed that there is underutilization of mRNAs in VSV-infected cells. The accumulation of 80S ribosomes and previous studies with UV-irradiated VSV (96, 149) lead them to postulate the existance of a virus-induced or specified nonspecific inhibitor of protein synthesis initiation that inhibits both viral and host mRNAs. Such an inhibitor would affect the initiation factor eIF-2 (21). Schnitzlein et al (113) used VSV DI-particles to reduce the level of wild-type mRNA synthesis. As a result mRNA synthesis was reduced considerably, but host protein synthesis inhibition was little affected.

The synthesis of ribosomal proteins and rRNAs is also inhibited in VSV-infected cells. This leads to inhibition of ribosome assembly, which further limits both host and virus protein synthesis (70). This must be true also for the other components of the translational machinery such as initiation—and elongation factors and so forth.

Models Based on VSV-Specific Functions

UV-inactivated VSV was used very early in the studies of host inhibition; infectivity was lost with low doses, but the ability to shut off protein synthesis was more resistant. Thus it was not surprising that virion proteins were thought to cause protein synthesis inhibition (11, 66, 156).

UV-inactivated VSV, which expressed only N-protein in the infected cells, still inhibited host protein synthesis (96), under conditions in which only partial transcription and no genome replication took place. If the cells are infected with transcription-negative ts-mutants at the restrictive temperature, even with high multiplicities, no host protein synthesis inhibition takes place (90, 95, 98, 103, 104, 129).

These mutants belong to complementation group I, which is assigned to the L-protein gene (104). Transcription-negative mutants, such as G114, have a

ts-defect in the virion enzyme and thus are unable to carry out the primary transcription step in the infected cells, incubated at the restrictive temperature. Other mutants of the same complementation group show a variable degree of transcription but are unable to replicate their genome. One of these, O5, shows inhibition of host protein synthesis and also cell-killing activity (95).

A mutant G22 of complementation group II, respective to NS-protein that shows primary transcription but no replication of RNA, inhibited host protein synthesis at the restrictive temperature to the same extent as the wild-type VSV. G22 synthesized 30–50% RNA as compared to the wild type. Some mutants of complementation group IV, respective to N-protein, show also reduced rates of transcription (91) and inhibit host protein synthesis slowly (90).

The VSV-specific function(s) responsible for the host protein synthesis shut-off have been narrowed by UV-irradiation using different doses (28). VSV, exposed to 400–600 ergs/mm^2, showed an increased host protein synthesis inhibition. In infected cells or lysates of them, the only virus-specific protein synthesized was N-protein. With higher doses, 2000–4000 ergs/mm^2, there was still inhibition of host protein synthesis of the order of 20–40% of the maximum. This could be eliminated by doses over 18,000 ergs/mm^2 or by heating the virus at 50°C.

The target size for the residual inhibitory activity was calculated to be 42 ± 12 nucleotides, the size of the plus-leader RNA. Interestingly, only the larger target was found by Stanner and coworkers (129) in the R1 mutant, which has a reduced capacity to inhibit host protein synthesis (90).

Finally, infection with DI-LT VSV, which lacks most of the L-protein gene (for review see 107), kills the host cells (94). The defective genome is transcribed by the virion transcriptase, which is derived from the helper (standard) VSV. No replication can take place, only primary translation, which yields N-, NS-, M-, and G-proteins but no L-protein.

Summarizing the ts-mutant and UV studies it can be stated that: (*a*) primary transcription is required and is sufficient to produce host protein synthesis inhibition; (*b*) partial primary transcription yielding mRNA for N-protein suffices for the inhibition; (*c*) when this function of the N-gene is abolished by UV irradiation, a very UV-resistant inhibition remains that has a target size of the plus-leader RNA.

In Vitro Studies

Lysates from VSV-infected L-cells prepared 4 hr after infection were less active than lysates from mock-infected cells (69). The activity could be restored by addition of a salt wash from ribosomes either from uninfected or infected cells or by addition of partially purified eIF-2 from uninfected cells (21, 135). The authors suggest that in VSV-infected cells, eIF-2 may have

reduced ability to form the ternary complex, perhaps due to modifications induced by the virus infection.

Experiments with VSV-infected *Aedes albopictus*–lysates (46, 47) have shown again that lysates from virus-infected cells are less efficient in supporting endogenous or exogenous mRNA translation. There was a decreased phosphorylation in vitro of a ribosomal protein component by γ (^{32}P)-ATP and formation of 80S initiation complex. These activities could be restored by adding a heat-labile component with S100 fraction, which was derived from uninfected cells (47).

INHIBITION OF HOST RNA AND DNA SYNTHESIS BY VSV

Inhibition of host-cell nucleic acid synthesis by active and UV-inactivated VSV has been studied during the past 20 years (45, 66, 99, 144, 145, 148, 151, 155, 156). The degree of inhibition varies in different cell lines as do the kinetics of inhibition. At best the inhibition is 80–90% at 3–4 hr after infection. Polymerase II is inhibited more efficiently than polymerases I and III (144).

The intriguing observation that the inhibition of host RNA synthesis can be obtained by UV-irradiated virus (66) has been difficult to understand. While the infectivity is lost by 67% with a dose of 100 ergs/mm^2, the viral in vitro transcriptase activity is lost by 67% with about 1000 ergs/mm^2, (8, 145). The transcription of N-gene and translation of N-protein can be eliminated with about 1600 ergs/mm^2. About 20 times this dose was required to eliminate the inhibitory activity of host RNA synthesis (145). Wu & Lucas-Lenard (151) concluded that the transcription of N-gene is required for the inhibitory activity.

An interesting experiment has been recently reported by McGowan et al (99). The plus-leader RNA was isolated and added to an in vitro transcription system containing SV40 DNA, or a hybrid plasmid containing adenovirus late promoter or a plasmid with adenovirus-associated RNA genes. In all cases the added-leader RNA caused a clear inhibition of transcription of the added genes whether transcribed by polymerase II or polymerase III (adeno-associated genes).

If this observation can be verified by others and extended to the infected cells, it would explain the results obtained by UV-irradiated VSV (100). Kranz & Reichmann (83) used double-infection with VSV and SV40 to study the mechanism of DNA synthesis inhibition in monkey kidney cells. They concluded that the inhibition of SV40 DNA synthesis was an indirect result of inhibition of protein synthesis. McGowan & Wagner (100), on the other hand, think that a small RNA transcript may be responsible for the inhibition of DNA synthesis.

SUMMARY OF VSV

Lodish & Porter provided convincing evidence that competition between viral and cellular mRNAs would easily explain the observed inhibition. However, there is a considerable amount of evidence that seems to contradict their conclusion. Infection with UV-inactivated VSV wild-type and ts mutants as well as DI-LT results in inhibition of the host protein synthesis. They display only a primary transcription and translation and no genome replication for different reasons (UV damage, ts defect, or deletion). The minimum requirement seems to be the synthesis of the plus-leader RNA together with the N-protein mRNA.

The recent results by Kolakofsky and his collaborators suggest that the N-protein regulates the genome replication by an "attenuation" mechanism by binding to the plus-leader RNA during transcription, thus preventing the termination and reinitiation usually occuring during transcription. This shifts the complex from transcription to replication, i.e. synthesis of full length antigenome (13, 14), which is used as template for the synthesis of more minus strands. Both the genome and the antigenome consume N-proteins, because they are always found as RNPs, even as mRNPs (111).

What happens to the N-protein once the events are arrested at primary transcription and translation and there is no suitable acceptor as in normal infection? What happens to the leader RNA in the absence of synthesis of N-protein, as is the case after heavy UV irradiation of the input virus? In normal infection, N-protein is bound to the leader RNA. Are the leaders transported to the nucleus, where they interfere with transcription and possibly also with DNA replication? In normal VSV-infected cells, the situation is different. It may be that the host shut-off under normal conditions takes place by competition rather than by virus-specific functions, which can be revealed only under artificial experimental conditions.

OTHER VIRUSES

Reoviruses

Reoviruses consist of ten double-stranded RNA segments in a double capsid (core and outer capsid). The structure and replication of reovirus has been solved to a great extent and reviewed extensively (37, 72, 73). The virions have a transcriptase that is able to synthesize capped, single-stranded mRNAs both in vitro and in vivo (154). Upon infection the outer capsid is removed by proteolytic activity of the cells and the subviral, parental particle (SVP) is released into the cytoplasm, starting the primary transcription of 10 mRNAs from the ten double-stranded genome segments. Part of the transcripts are used as templates for the synthesis of a second strand, which takes place in

progeny subviral particles. These in turn start the secondary transcription of mRNAs.

There is no dramatic shut-off of protein synthesis in reovirus-infected cells (153). Rather, there is a shift from cellular to viral protein synthesis late in infection (72, 73). Early in infection (up to 4–6 hr) capped and methylated mRNAs are transcribed from the parental SVPs, which are translated in lysates from normal cells (118, 119, 152). Later in infection uncapped mRNAs are synthesized. They are not translated in lysates from normal cells, but are translated well in lysates from reovirus-infected cells (120). Modifications of the translational apparatus in reovirus-infected cells are not known so far.

Reovirus type 3 inhibits the host-cell DNA synthesis (33, 72) while type 1 does not (115). By elegant recombination analysis Sharpe & Fields showed that the inhibitory gene segment of type 3 was S1, which codes for the hemagglutinin protein that is the outer capsid protein σ_1. Since UV-irradiated reovirus type 3 can still inhibit cellular DNA synthesis, it could not be established with certainty whether the inhibition was due to the parental virion or a primary translational product of the parental SVP in the infected cell (116).

SUMMARY

The studies with poliovirus have revealed that cells have a full capacity to translate uncapped mRNA, although their own mRNAs are capped and methylated. These observations have been confirmed by translation studies in fractionated systems in vitro. In poliovirus-infected cells, and in cell extracts prepared from them, a well-documented loss of capacity to translate capped mRNAs has been shown. This is due to the dissociation of cap-binding protein(s) from the initiation factor eIF-3.

It has not been possible to show which virus-specific protein, if any, is responsible for this dissociation. It may be that once not used, the cap-binding protein is excluded passively from its normal association cycle with eIF-3, rendering the cells incapable of translating capped mRNAs.

Late in reovirus infection a similar phenomenon is observed. The secondary transcription products of reovirus subviral particles are uncapped mRNAs, which are synthesized in abundance. Their capping and methylation is prevented in the SVPs due to a masking of the respective enzymes (119). The inability to translate capped mRNAs in lysates prepared from reovirus-infected cells, late in infection, may be due to a similar mechanism, as in poliovirus-infected cells. Simply, a large excess of uncapped mRNA competes with initiation factors (15, 16, 52, 141) without specific need for the cap-binding protein(s), which becomes dissociated from the eIF-3 and loses its activity.

Inhibition of host protein synthesis, whether due to simple competition or specified by a viral gene product, leads apparently to a shortage of initiation

factors and ribosomes, causing the cessation of translation of both capped and uncapped mRNA, and finally to cell death.

The studies with UV-inactivated VSV, its temperature-sensitive mutants and defective-interfering LT-particles clearly suggest that inhibition of host-cell macromolecular synthesis can be induced by mechanisms that do not operate during normal VSV-infection. It may be that similar phenomena occur in guanidine-treated poliovirus-infected cells. In these cells the host protein synthesis is inhibited as a result of the translation of a few hundred incoming viral RNAs.

It would be interesting to know whether the cap-binding protein is dissociated from initiation factors under these conditions. If not, another mechanism has to be revealed, which again may not be manifest during normal poliovirus infection.

The inhibition of host RNA and DNA synthesized by various viruses has been less intensively studied than inhibition of protein synthesis. Apparently, good in vitro systems are needed to solve these difficult problems (26, 99).

Literature Cited

1. Abraham, G., Banerjee, A. K. 1976. *Proc. Natl. Acad. Sci. USA* 73:1504–8
2. Alonso, M. A., Carrasco, L. 1982. *EMBO J.* 1:913–17
3. Alonso, M. A., Carrasco, L. 1982. *J. Gen. Virol.* 61:15–24
4. Apriletti, J. W., Penhoet, E. E. 1974. *Virology* 61:597–601
5. Apriletti, J. W., Penhoet, E. E. 1978. *J. Biol. Chem.* 253:603–11
6. Atkins, G. J. 1976. *Virology* 71:593–97
7. Baglioni, C., Maroney, P. A., Simli, M. 1979. In *The Molecular Biology of the Picornaviruses*, ed. R. Perez-Beroff, pp. 101–11. New York: Plenum
8. Ball, L. A., White, C. N. 1976. *Proc. Natl. Acad. Sci. USA* 73:442–46
9. Baltimore, D. 1969. In *The Biochemistry of Viruses*, ed. H. B. Levy, pp. 101–76. New York: Dekker
10. Banerjee, A. K. 1980. *Bacteriol. Rev.* 44:175–205
11. Baxt, B., Bablanian, R. 1976. *Virology* 72:383–92
12. Bishop, D. H. L., Shope, R. E. 1979. In *Comprehensive Virology*, Vol. 14, ed. H. Fraenkel-Conrat, R. R. Wagner, pp. 1–156. New York: Plenum
13. Blumberg, B. M., Giorgi, C., Kolakofsky, D. 1983. *Cell* 32:559–67
14. Blumberg, B. M., Leppert, M., Kolakofsky, D. 1981. *Cell* 23:837–45
15. Brendler, T., Godefroy-Colburn, T., Carlill, R. D., Thach, R. E. 1981. *J. Biol. Chem.* 256:11747–54
16. Brendler, T., Godefroy-Colburn, T., Yu, S., Thach, R. E. 1981. *J. Biol. Chem.* 256:11755–61
17. Brown, B. A., Ehrenfeld, E. 1980. *Virology* 103:327–39
18. Brown, D., Hansen, J., Ehrenfeld, E. 1980. *J. Virol.* 34:573–75
19. Carrasco, L., Smith, A. E. 1976. *Nature* 264:807–9
20. Celma, M. L., Ehrenfeld, E. 1974. *Proc. Natl. Acad. Sci. USA* 71:2440–44
21. Centrella, M., Lucas-Lenard, J. 1982. *J. Virol.* 41:781–91
22. Choppin, P. W., Compans, R. W. 1975. See Ref. 12, pp. 95–178
23. Cole, C. N., Baltimore, D. 1973. *J. Mol. Biol.* 76:325–43
24. Compans, R. W., Choppin, P. W. 1975. See Ref. 12, pp. 179–252
25. Contreras, G., Summers, D. F., Maizel, J. V., Ehrenfeld, E. 1973. *Virology* 53: 120–29
26. Crawford, N., Fire, A., Samuels, M., Sharp, P. A., Baltimore, D. 1981. *Cell* 27:555–61
27. Dasgupta, A. 1983. *Virology* 128:252–59
28. Dunigan, D. D., Lucas-Lenard, J. M. 1983. *J. Virol.* 45:618–26
29. Edery, I., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., et al. 1983. *J. Biol. Chem.* 258:11398–403
30. Ehrenfeld, E. 1982. *Cell* 28:435–36
31. Ehrenfeld, E., Hunt, T. 1971. *Proc. Natl. Acad. Sci. USA* 68:1075–78

32. Ehrenfeld, E., Lund, H. 1977. *Virology* 80:297–308
33. Ensminger, W. D., Tamm, I. 1969. *Virology* 39:935–38
34. Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N., Hershey, J. W. B. 1982. *J. Biol. Chem.* 257:14806–10
35. Fellner, P. 1979. See Ref. 7, pp. 25–47
36. Fernandez-Munoz, R., Darnell, J. E. 1976. *J. Virol.* 18:719–26
37. Fields, B. N., Greene, M. I. 1982. *Nature* 300:19–23
38. Flanegan, J. B., Baltimore, D. 1979. *J. Virol.* 29:352–60
39. Flores-Otero, G., Fernandez-Tomas, C., Gariglio-Vidal, P. 1982. *Virology* 116:619–28
40. Gallione, C. J., Greene, J. R., Iverson, L. E., Rose, J. K. 1981. *J. Virol.* 39:529–35
41. Gallwitz, D., Traub, U., Traub, P. 1977. *Eur. J. Biochem.* 81:387–93
42. Garoff, H., Kondor-Koch, C., Riedel, H. 1982. *Curr. Top. Microbiol. Immunol.* 99:1–50
43. Garry, R. F., Bishop, J. M., Parker, S., Westbrook, K., Lewis, G., et al. 1979. *Virology* 96:108–20
44. Garry, R. F., Westbrook, K., Waite, M. R. F. 1979. *Virology* 99:179–82
45. Genty, N. 1975. *J. Virol.* 15:8–15
46. Gillies, S., Stollar, V. 1981. *J. Biol. Chem.* 256:13188–92
47. Gillies, S., Stollar, V. 1982. *Mol. Cell. Biol.* 2:1174–86
48. Glanville, N., Lachmi, B. 1977. *FEBS Lett.* 81:399–402
49. Glanville, N., Ulmanen, I. 1976. *Biochem. Biophys. Res. Commun.* 71:393–99
50. Glanville, N., Morser, J., Uomala, P., Kääriäinen, L. 1976. *Eur. J. Biochem.* 64:167–75
51. Glanville, N., Ranki, M., Morser, J. Kääriäinen, L., Smith, A. E. 1976. *Proc. Natl. Acad. Sci. USA* 73:3059–63
52. Godefroy-Colburn, T., Thach, R. E. 1981. *J. Biol. Chem.* 256:11762–73
53. Golini, F., Thach, S. S., Birge, C. H., Safer, B., Merrick, W. C., et al. 1976. *Proc. Natl. Acad. Sci. USA* 73:3040–44
54. Gray, M. A., Micklem, K. J., Brown, F., Pasternak, C. A. 1983. *J. Gen. Virol.* 64:1449–56
55. Grifo, J. A., Tahara, S. M., Leis, J. P., Morgan, M. A., Shatkin, A. J., et al. 1982. *J. Biol. Chem.* 257:5246–52
56. Hand, R., Oblin, C. 1977. *J. Gen. Virol.* 37:349–58
57. Hanecak, R., Semler, B. L., Anderson, C. W., Wimmer, E. 1982. *Proc. Natl. Acad. Sci. USA* 79:3973–77
58. Hansen, J., Ehrenfeld, E. 1981. *J. Virol.* 38:438–45
59. Hansen, J., Etchison, D., Hershey, J. W. B., Ehrenfeld, E. 1982. *J. Virol.* 42: 200–07
60. Hay, A. J., Skehel, J. J., Burke, D. C. 1968. *J. Gen. Virol.* 3:175–84
61. Helentjaris, T., Ehrenfeld, E. 1977. *J. Virol.* 21:259–67
62. Helentjaris, T., Ehrenfeld, E. 1978. *J. Virol.* 26:510–21
63. Helentjaris, T., Ehrenfeld, E., Brown-Luedi, M. L., Hershey, J. W. B. 1979. *J. Biol. Chem.* 254:10973–78
64. Hewlett, M. J., Rose, J. K., Baltimore, D. 1976. *Proc. Natl. Acad. Sci. USA* 73:327–30
65. Holland, J. 1963. *J. Mol. Biol.* 8:574–81
66. Huang, A. S., Wagner, R. R. 1965. *Proc. Natl. Acad. Sci. USA* 54:1579–84
67. Hunt, T., Ehrenfeld, E. 1971. *Nature New Biol.* 230:91–94
68. Jacobson, M. F., Baltimore, D. 1968. *Proc. Natl. Acad. Sci. USA* 61:77–84
69. Jaye, M. C., Godchaux, W. III, Lucas-Lenard, J. 1982. *Virology* 16:148–62
70. Jaye, M., Wu, F.-S., Lucas-Lenard, J. M. 1980. *Biochem. Biophys. Acta* 606:1–12
71. Jen, G., Morgan-Detjen, B., Thach, R. E. 1980. *J. Virol.* 35:150–56
72. Joklik, W. K., 1974. See Ref. 12, pp. 231–334
73. Joklik, W. K. 1981. *Microbiol. Rev.* 45:483–501
74. Kääriäinen, L., Pesonen, M. 1982. In *Glycoconjugates*, Vol. IV, ed. M. I. Horowitz, pp. 191–242. New York: Academic
75. Kääriäinen, L., Söderlund, H. 1978. *Curr. Top. Microbiol. Immunol.* 82:15–69
76. Keränen, S., Kääriäinen, L. 1974. *Acta Pathol. Microbiol. Scand. Sect. B* 82: 810–20
77. Keränen, S., Kääriäinen, L. 1975. *J. Virol.* 16:388–96
78. Keränen, S., Kääriäinen, L. 1979. *J. Virol.* 32:19–29
79. Keränen, S., Ruohonen, L. 1983. *J. Virol.* 47:505–15
80. Kitamura, N., Semler, B. L., Rothberg, P. G., Larsen, G. R., Adler, C. J., et al. 1981. *Nature* 291:547–53
81. Koizumi, S., Simizu, B., Hashimoto, K., Oya, A., Yamada, M. 1979. *Virology* 94:314–22
82. Koizumi, S., Simizu, B., Ishida, I., Oya, A., Yamada, M. 1979. *Virology* 98:439–47
83. Kranz, D., Reichmann, M. E. 1983. *Virology* 128:418–28
84. Krug, R. M. 1981. In *Initiation Signals*

in Viral Gene Expression, ed. A. J. Shatkin, Vol. 93, pp. 125–49. Berlin: Springer
85. Lacal, J. C., Carrasco, L. 1983. *J. Gen. Virol.* 64:787–93
86. Lachmi, B., Kääriäinen, L. 1977. *J. Virol.* 22:142–49
87. Lee, K. A. W., Sonenberg, N. 1982. *Proc. Natl. Acad. Sci. USA* 79:3447–51
88. Leppert, M., Rittenhouse, L., Perrault, J., Summers, D. F., Kolakofsky, D. 1979. *Cell* 18:235–45
89. Lodish, H. F., Porter, M. 1980. *J. Virol.* 36:719–33
90. Lodish, H. F., Porter, M. 1981. *J. Virol.* 38:504–17
91. Lodish, H. F., Weiss, R. A. 1979. *J. Virol.* 30:177–89
92. Lucas-Lenard, J. M. 1979. See Ref. 7, pp. 73–99
93. Lust, G., 1966. *J. Bacteriol.* 91:1612–17
94. Marcus, P. I., Sekellick, M. J., Johnson, L. P., Lazzarini, R. A. 1977. *Virology* 82:242–46
95. Marvaldi, J., Lucas-Lenard, J., Sekellick, M. J., Marcus, P. I. 1977. *Virology* 79:267–80
96. Marvaldi, J., Sekellick, M. J., Marcus, P. I., Lucas-Lenard, J. 1978. *Virology* 84:127–33
97. Matlin, K. S., Reggio, H., Helenius, A., Simons, K. 1982. *J. Mol. Biol.* 156: 609–31
98. McAllister, P. E., Wagner, R. R. 1976. *J. Virol.* 18:550–58
99. McGowan, J. J., Emerson, S. U., Wagner, R. R. 1982. *Cell* 28:325–33
100. McGowan, J. J., Wagner, R. R., 1981. *J. Virol.* 38:356–67
101. Otto, M. J., Lucas-Lenard, J. 1980. *J. Gen. Virol.* 50:293–307
102. Porter, A., Carey, N. H., Fellner, P. 1974. *Nature* 248:675–78
103. Pringle C. R., 1977. In *Comprehensive Virology*, Vol. 9, ed. H. Fraenkel-Conrat, R. R. Wagner, pp. 239–89. New York: Plenum
104. Pringle, C. R. 1982. *Arch. Virol.* 72:1–34
105. Racaniello, V. R., Baltimore, D. 1981. *Proc. Natl. Acad. Sci. USA* 78:4887–91
106. Ranki, M., Ulmanen, I., Kääriäinen, L. 1979. *FEBS Lett.* 108:299–302
107. Reichmann, M. E., Schnitzlein, W. M. 1979. *Curr. Top. Microbiol. Immunol.* 86:124–68
108. Rose, J. K. 1980. *Cell* 19:415–21
109. Rose, J. K., Gallione, C. J. 1981. *J. Virol.* 39:519–28
110. Rose, J. K., Trachsel, H., Leong, K., Baltimore, D. 1978. *Proc. Natl. Acad. Sci. USA* 77:770–74
111. Rosen, C. A., Ennis, H. L., Cohen, P. S. 1982. *J. Virol.* 44:932–38
112. Rubio, C., Kolakofsky, C., Hill, V. M., Summers, D. F. 1980. *Virology* 105:123–35
113. Schnitzlein, W. M., O'Banion, M. K., Poirot, M. K., Reichmann, M. E. 1983. *J. Virol.* 45:206–14
114. Schwartz, L. B., Lawrence, C., Thach, R. E., Roeder, R. G., 1974. *J. Virol.* 14:611–19
115. Sharpe, A. H., Fields, B. N. 1981. *J. Virol.* 38:389–92
116. Shaw, J. E., Cox, D. C. 1973. *J. Virol.* 12:704–10
117. Simizu, B., Wagatsuma, M, Oya, A., Hanaoka, F., Yamada, M. 1976. *Arch. Virol.* 51:251–61
118. Skup, D., Millward, S. 1980. *Proc. Natl. Acad. Sci. USA* 77:152–56
119. Skup, D., Millward, S. 1980. *J. Virol.* 34:490–96
120. Skup, D., Zarbl, H., Millward, S. 1981. *J. Mol. Biol.* 151:35–55
121. Smith, A. E., Wheeler, T., Glanville, N., Kääriäinen, L. 1974. *Eur. J. Biochem.* 49:101–10
122. Söderlund, H., Ulmanen, I. 1977. *J. Virol.* 24:907–9
123. Sonenberg, N. 1981. *Nucl. Acid. Res.* 9:1643–56
124. Sonenberg, N., Shatkin, A. J. 1977. *Proc. Natl. Acad. Sci. USA* 74:4288–92
125. Sonenberg, N., Merrick, N. C., Morgan, M. A., Shatkin, A. J. 1978. *Proc. Natl. Acad. Sci. USA* 75:4843–47
126. Sonenberg, N., Rupprecht, K. M., Hecht, S. M., Shatkin, A. J. 1979. *Proc. Natl. Acad. Sci. USA* 76:4345–49
127. Sonenberg, N., Trachsel, H., Hecht, S., Shatkin, A. J. 1980. *Nature* 285:331–33
128. Sonenberg, N., Guertin, D., Cleveland, D., Trachsel, H. 1981. *Cell* 27:563–72
129. Stanners, C. P., Francoeur, A. M., Lam, T. 1977. *Cell* 11:273–81
130. Steiner-Pryor, A, Cooper, P. D. 1973. *J. Gen. Virol.* 21:215–25
131. Strauss, J. H., Strauss, E. G. 1977. In *The Molecular Biology of Animal Viruses*, ed. D. P. Nayak, pp. 111–16 New York: Dekker
132. Strauss, E. G., Strauss, J. H. 1983. *Curr. Top. Microbiol. Immunol.* 105:1–98
133. Tahara, S. M., Morgan, M. A., Grifo, J. A., Merrick, W. C., Shatkin, A. J. 1982. In *Interaction of Translational and Transcriptional Controls in the Regulation of Gene Expression*, ed. M. Grunberg-Manago, B. Safer, pp. 359–72. New York: Elsevier
134. Tahara, S. M., Morgan, M. A., Shatkin, A. J. 1981. *J. Biol. Chem.* 256:7691–94

135. Thomas, J. R., Wagner, R. R. 1983. *Biochemistry* 22:1540–46
136. Trachsel, H., Sonenberg, N., Shatkin, A. J., Rose, J. K., Leong, K. 1980. *Proc. Natl. Acad. Sci. USA* 77:770–74
137. Tuomi, K., Kääriäinen, L., Söderlund, H. 1975. *Nucl. Acid Res.* 2:555–65
138. Ulmanen, I., Söderlund, H., Kääriäinen, L. 1976. *J. Virol.* 20:203–10
139. Ulmanen, I., Söderlund, H., Kääriäinen, L. 1979. *Virology* 99:265–76
140. Wagner, R. R. 1975. In *Comprehensive Virology*, Vol. 4, ed. H. Fraenkel-Conrat, R. R. Wagner, pp. 1–93. New York: Plenum
141. Walden, W. E., Godefroy-Colburn, T., Thach, R. E. 1981. *J. Biol. Chem.* 256:11739–46
142. Van Steeg, H., Pranger, M. H., van der Zeijst, B. A. M., Benne, R., Voorma, H. O. 1979. *FEBS Lett.* 108:292–98
143. Van Steeg, H., Thomas, A., Verbeek, S., Kasperaitis, M., Voorma, H. et al. 1981. *J. Virol.* 38:728–36
144. Weck, P. K., Wagner, R. R. 1978. *J. Virol.* 25:770–80
145. Weck, P. K., Carrol, A. R., Shattuck, D. M. Wagner, R. R. 1979. *J. Virol.* 30: 746–53
146. Wengler, G. 1980. In *Togaviruses*, ed. R. W. Schlesinger, pp. 459–72. New York: Academic
147. Wengler, G., Wengler, G. 1976. *J. Virol.* 17:10–19
148. Wertz, G. W., Younger, J. S. 1970. *J. Virol.* 6:476–84
149. Wertz, G. W., Younger, J. S. 1972. *J. Virol.* 9:85–89
150. Wimmer, E. 1979. See Ref. 7, pp. 175–90
151. Wu, F.-S., Lucas-Lenard, J. M. 1980. *Biochemistry* 19:804–10
152. Zarbl, H., Skup, D., Millward, S. 1980. *J. Virol.* 34:497–505
153. Zweerink, H. J., Joklik, W. K. 1970. *Virology* 41:501–18
154. Yamakawa, M., Shatkin, A. J., Furuichi, Y. 1982. *Virology* 118:157–68
155. Yamazaki, S., Wagner, R. R. 1970. *J. Virol.* 6:421–29
156. Yaoi, Y., Mitsui, H., Amano, M. 1970. *J. Gen. Virol.* 8:165–72

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THE GENETICS OF THE GONOCOCCUS

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INTRODUCTION

Neisseria gonorrhoeae, a gram-negative coccus, is the causative agent of the human sexually transmitted disease gonorrhea. Our knowledge of the gonococcus has undergone a remarkable transition in recent years. Many workers have begun to use genetic approaches to try to address questions about the pathogenesis of infection and about the regulation and function of specific gonococcal components. In this review, we will first survey the genetic systems that are available to study gonococci and then consider the ways these systems have been exploited in the study of different aspects of gonococcal infection.

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GENETIC SYSTEMS IN THE GONOCOCCUS

Transformation

COMPETENCE Gonococci are unusual in the ease with which they can be genetically transformed with naked transforming DNA. Recently isolated gonococcal strains nearly always are transformable (28, 152). However, competence for genetic transformation is much greater in piliated (Pil^+) colonial forms of the gonococcus than in their isogenic nonpiliated (Pil^-) colonial variants (152). Typical transformation frequencies for chromosomal markers in Pil^+ recipients are about 1×10^{-3}, whereas in Pil^- recipients transformation frequencies seldom exceed 1×10^{-7} (152). Pil^+ cells are capable of taking up transforming DNA into a DNase-resistant state, whereas Pil^- cells are not (9, 36).

Using the formula developed by Goodgal & Herriott (45) for estimating competence in a population of cells, 35–100% of Pil^+ cells are competent for transformation (9). Competence in Pil^+ cells is expressed constitutively, with no convincing evidence either for development of waves of competence or for soluble competence factors (9). Phenotypic expression of competence in vitro requires only a utilizable energy source (glucose) and cations, preferably a divalent cation such as Mg^{2+} or Ca^{2+} (9). It has been claimed that competence is increased by presence of a 24-Mdal plasmid (114), but we have found no effect of resident 2.6 or 24-Mdal plasmids on competence of isogenic strains (G. Biswas & P. F. Sparling, unpublished data).

Although nearly all recent clinical isolates are competent, the competent state is rapidly lost in vitro, coincident with the high-frequency transition from predominantly Pil^+ to Pil^- cells on unselected serial culture (67, 152, 167). It is possible that pili are directly related to ability to take up transforming DNA, perhaps by acting as a receptor or by helping to create a channel through the outer membrane. To date, however, no evidence is available to support a role of pili in DNA uptake, and it is equally possible that presence of pili merely reflects a complicated phenotype that includes competence for DNA uptake. Regardless of the precise role of pili in DNA uptake, their role in mediating early steps in pathogenesis of genital infection (20, 35, 74, 160) exerts strong selective pressures for maintenance of the Pil^+ (competent) phase.

Because gonococci presumably are highly competent in vivo, and gonococcal DNA released by autolysis and recovered from patients is biologically active in transformation (60, 65), it would not be surprising if gonococci exchange DNA in vivo by transformation. Indeed, it has been suggested that transformation might be the principal means of exchange of chromosomal DNA between gonococci in nature (46), inasmuch as there are no known gonococcal bacteriophage (44), and gonococcal conjugal plasmids fail to

detectably mobilize chromosomal genes (6, 114, 159). Mixed culture of different gonococcal strains in vitro rapidly results in high frequencies of transformation (136).

Transformation also might be a means of exchanging genes between gonococci and other Neisseria. Gonococci and meningococci show considerable DNA homology, and interspecific transformation between gonococci and meningococci is quite efficient for many genes (153). Ison et al recently demonstrated that meningococcal DNA could transform gonococci for certain characteristics commonly used to differentiate these organisms, including ability to utilize maltose, and suggested that transformation in vivo theoretically could produce interspecies hybrids that would pose problems for diagnostic laboratories (58).

DNA UPTAKE The mechanisms of DNA uptake in gonococci are understood relatively poorly, but there are several obvious similarities with the *Haemophilus* transformation system. Transforming DNA is taken up by gonococci (10) and *Haemophilus* (159a) as double strands and remains double-stranded presumably until shortly before recombination with the recipient chromosome. Competent gonococci (36, 46, 95), like *Haemophilus* (148), preferentially take up homologous DNA; heterologous DNA is loosely bound to the cell surface, but never passes through the outer membrane into a DNase-resistant state (36, 46). The DNA sequence or structure required by gonococci for uptake is unknown, but apparently is different from the analogous 11-mer described by Sisco & Smith (148) in *Haemophilus influenzae* because *H. influenzae* DNA does not inhibit uptake of gonococcal DNA by competent gonococci (46, 95).

It has been suggested that one or more of the numerous gonococcal DNA methylases could promote recognition by gonococci of their own DNA (81), but because a hybrid shuttle vector grown in *E. coli* transforms gonococci approximately as well as when the plasmid is prepared from a gonococcal host (157), methylation probably is not necessary for specific uptake. The nature of the putative receptor for recognizing homologous DNA is unknown. Dougherty et al demonstrated that DNA uptake by competent gonococci is followed by a period of over 20 minutes of relative inability to be transformed by another molecule of transforming DNA (36), which may explain the low frequencies ($<0.2\%$) of simultaneous transformation of competent gonococci by two independent molecules of transforming DNA carrying unlinked markers (149).

PLASMID TRANSFORMATION Transformation of gonococci by plasmid DNA has been the subject of much recent interest, partly in anticipation of using recombinant plasmids for a variety of molecular genetic purposes. In general, plasmid transformation in gonococci is inefficient. The naturally occurring 4.7-Mdal penicillinase-producing (Pc^r) plasmid pFA3 results in ≤ 1

$\times 10^{-7}$ Pc^r transformants per μg plasmid DNA (151). Transformation efficiencies are increased about 10-fold in hybrids of pFA3 containing gonococcal DNA, apparently because these contain necessary DNA sequences for efficient DNA uptake (46). Plasmids recovered from transformants frequently suffer deletions (8, 151) unless the recipient strain contains a homologous plasmid; presence of a homologous recipient plasmid also markedly (1000-fold) increases plasmid transformation efficiencies (8).

These effects are very similar to the marker rescue phenomena described in *Bacillus subtilis* (30) and other organisms and are presumed to result from linearization of the donor plasmid during entry, followed by recombination with the recipient plasmid ("rescue"). Both circular plasmid and linear chromosomal DNA must share some early steps in transformation, because most gonococcal mutants blocked in DNA uptake have similar effects on transformation by plasmid and chromosomal DNA (G. Biswas & P. F. Sparling, unpublished data).

Recently, Stein et al (145, 157, 158) created a hybrid of a gonococcal Pc^r plasmid that should be particularly useful for cloning gonococcal DNA in *E. coli*. This hybrid (pLES2) contains several unique restriction sites suitable for cloning in *lacZ* DNA, and also a separate region encoding a selectable Pc^r phenotype; it transforms *E. coli* readily, but transforms gonococci very poorly unless it contains inserted gonococcal DNA. Unfortunately, lack of documented recombination-deficient gonococcal strains hinders use of recombinant plasmids in complementation or related studies.

Conjugation

From 8% to 22% of unselected gonococci (113, 150), and a higher proportion of Pc^r gonococci (66, 113), possess a 24-Mdal conjugative plasmid that mobilizes transfer to other gonococci of plasmids (e.g. the 4.7- or 3.4-Mdal Pc^r plasmids) with high efficiency (39, 42, 77, 125, 150). As many as 10% of cells carrying the 24-Mdal plasmid may act as conjugal donors under suitable conditions (6), suggesting that the gonococcal conjugal system normally is not repressed. Efficiencies of conjugation are reduced if donors or recipients contain heat-modifiable proteins related to colonial opacity (6).

Attempts to visualize sex pili by electron microscopy were unsuccessful (S. To & C. Brinton, personal communication). The 24-Mdal conjugal plasmid apparently replicates only transiently in *E. coli* (42), which has made it impossible to classify it by incompatibility grouping. A physical map of the plasmid has been prepared (170); restriction maps of 24-Mdal plasmids isolated from different gonococcal strains are very similar, although they differ significantly in their efficiencies of mobilization of a Pc^r plasmid (150).

It would be useful if gonococci possessed a conjugal system for chromosomal gene transfer. A report of chromosomal gene transfer by the 24-Mdal plasmid (126) was met with enthusiasm, but subsequent efforts to confirm this report have been unsuccessful (6, 114, 159). Recombinants observed in the initial report probably resulted from low-frequency transformation between whole cells in a prolonged filter-disc mating, despite addition of DNAase at the onset of mating (6, 159).

Mutation

Relatively little work has been done on mutagenesis of gonococci. Although mutants have been reported after exposure to ultraviolet light (93), gonococci and meningococci lack systems for photoreactivation (22) and for error-prone repair (L. A. Campbell & R. E. Yasbin, personal communication), and ultraviolet mutagenesis undoubtedly is highly inefficient. Maximum mutation frequencies after ethyl methane sulfonate or nitrosoguanidine mutagenesis are about 3×10^{-4}.

Transposon mutagenesis has not been reported yet in gonococci, no doubt because ethical considerations prevent introduction of most antibiotic-resistance transposons. The only transposon that occurs naturally in gonococci is a portion of Tn2, and it is incapable of transposition because it contains only 40% of Tn2, including only one of the two terminal inverted repeats (41, 149). Future work would be aided considerably by success in mutagenesis with transposons carrying genes for resistance to agents other than antibiotics, or by agents such as Mu.

APPLICATIONS OF GENETICS TO STUDY OF THE BIOLOGY OF THE GONOCOCCUS

Strains from Disseminated Gonococcal Infection (DGI)

Strains of gonococci isolated from patients with DGI (the gonococcal bacteremia-dermatitis-arthritis syndrome) tend to share a number of phenotypic characteristics that are found less commonly among strains causing local uncomplicated infection. These characteristics include resistance to the bactericidal action of normal human serum (139), susceptibility to penicillin (181), and requirements for arginine (Arg), hypoxanthine (Hyx), and uracil (Ura) for growth (27, 78).

Also, these strains frequently possess a particular immunochemical type of Protein I of the outer membrane (19, 24) and almost always contain methyladenine in their DNA (79). The existence of such a constellation of properties in strains that cause a particular type of infection raises the question of whether these different phenotypes are the result of a single genetic event or whether they represent mutations or differences in multiple genes. This has been studied by Mayer et al (97) and Cannon et al (26), who used genetic transformation to

demonstrate that some of these properties are independent, involving separate genetic loci.

If the different properties of DGI strains are not the consequence of a single mutation, how can the frequent occurrence of strains with all of these properties be explained? One mechanism might be by derivation of these strains from a common ancestor. Several pieces of evidence have been interpreted as supporting this possibility, although none is definitive. In the transformation studies of Mayer et al (97), each of the tested DGI strains had identical genetic lesions leading to an Arg requirement (there was no recombination among the different strains resulting in prototrophy).

In contrast, several different lesions have been identified in strains causing local urogenital infection that can lead to each of the different auxotrophic requirements, including proline, methionine, arginine, and thiamine pyrophosphate (28, 73). Mayer et al argued that, if the Arg^-, Hyx^-, and Ura^- phenotypes are important for virulence of DGI strains, and if they arise by random mutations, one would expect to see several genetic lesions responsible for these requirements in DGI strains. They concluded that it is likely that the most prevalent type of DGI strain arose from a single ancestor.

An alternative model to explain the association of the family of traits in DGI strains is that there is strong selection in the host in favor of strains with these particular phenotypes. Catlin (29) suggested a possible explanation for the relationship between penicillin sensitivity and auxotrophic requirements: because penicillin kills only growing cells, penicillin treatment might enrich for cells with auxotrophic requirements for nutrients in limiting concentration in vivo. There may also be some selection for auxotrophy even in infections that are not treated with penicillin.

Kenyon (75) reported that passage of a proline-requiring strain in a normal mouse resulted in acquisition of additional nutritional requirements (Arg, Hyx, and Ura) similar to those found in strains causing DGI in humans. This illustrates the potential importance of selective mechanisms that operate in the host, which may lead to selection for multiple mutations in a single strain. In the absence of a complete understanding of such selective mechanisms, it is difficult to draw firm conclusions about the possible origins of DGI strains.

Antibiotic Resistance

PENICILLINASE Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) first were reported in 1976 (3, 121) and subsequently have been an increasingly severe problem. PPNG produce a TEM-1 type ß-lactamase (5) typical of many enteric bacteria and usually contain either a plasmid variously estimated as 3.2 to 3.4 Mdal, or one estimated as 4.4 to 4.7 Mdal (33, 39, 124, 151). Presence of one of these Pc^r plasmids results in inoculum-dependent resistance, with typical minimum inhibitory concentrations to penicillin or ampicillin of 10–100 $\mu g\ ml^{-1}$ (118, 171). Nearly all gonococci also carry a cryptic plasmid of 2.6 Mdal, and many also carry a 24-Mdal conjugative plasmid (7, 39, 42, 77, 113, 124, 150).

PPNG appeared to originate nearly simultaneously in Southeast Asia and in sub-Saharan Africa (118), but were rapidly spread throughout the world. Most PPNG isolates from Southeast Asia were moderately resistant to many other antibiotics, (presumably because of chromosomal mutations), nearly always carried the 4.7-Mdal Pc plasmid, and often contained the 24-Mdal conjugative plasmid (118, 171). In contrast, most isolates from Africa or Europe were less resistant to other antibiotics, contained the 3.4-Mdal Pc^r plasmid, and did not contain the 24-Mdal plasmid (118, 171).

African and Southeast Asian PPNG also differed in nutritional requirements on defined media ("auxotype") (118). These observations suggested occurrence of two related but separate clones of PPNG in different geographic regions. Subsequently, PPNG strains with various combinations of these phenotypes have appeared (66), probably due to conjugal spread of the Pc^r plasmids between gonococci. The 24-Mdal plasmid readily mobilizes transfer of the 4.7-Mdal Pc^r plasmid to other gonococci in an animal model (127).

PPNG are now a very serious clinical problem in many parts of the world. The prevalence of PPNG is increasing in Europe (99, 174) and in the United States, although less than one percent of all gonococci in the U.S. are ß-lactamase producers. Fortunately, several other effective therapies are available (51).

There has been considerable interest in the molecular structures and possible origins of the gonococcal Pc^r plasmids. There is no significant homology between the Pc^r plasmids and either the indigenous gonococcal 2.6-Mdal cryptic plasmid or the 24-Mdal conjugative plasmid (124). However, the gonococcal Pc^r plasmids are very closely related to each other (33, 124, 151) and to similar Pc^r plasmids in *H. influenzae*, *H. parainfluenzae*, and *H. ducreyi* (16, 87, 124, 155).

Recently, ß-lactamase-producing isolates of *H. ducreyi*, the causative agent of chancroid, have been shown to carry plasmids almost identical to the gonococcal Pc^r plasmids, except the *H. ducreyi* plasmids contain the entirety of the Pc^r transposon Tn2 (17, 102); the gonococcal plasmids contain only the right-hand 40% of Tn2 (41). An isolate of *H. parainfluenzae* has been characterized with a 4.7-Mdal Pc^r plasmid that is identical to the similarly sized gonococcal plasmid (155). Both *H. parainfluenzae* and *H. ducreyi* can conjugally transfer their Pc^r plasmids to gonococci (102, 155), and gonococci with a 24-Mdal conjugal plasmid can mobilize transfer of *Haemophilus* Pc^r plasmids (42).

These observations do not prove the origins of the gonococcal Pc^r plasmids but are compatible with the concept that these plasmids are being transferred

between gonococci and *Haemophilus* in nature. A recent report of a genital isolate of *N. meningitidis* with a gonococcal-type Pc^r plasmid (34) is alarming, strongly suggesting the need for continued active surveillance for Pc^r meningococci.

CHROMOSOMAL RESISTANCE Fortunately, plasmid-mediated antimicrobial resistance in gonococci presently is limited to production of ß-lactamase, although it would be surprising if other types of plasmid or transposon-borne resistance do not occur in the future. Gonococci with a variety of mutations to resistance to multiple antimicrobials have been selected gradually over the past few decades, however, particularly in developing regions of the world where antibiotics often are available without prescription (129). Many of the mutations responsible for non-ß-lactamase–mediated resistance have been shown to be chromosomal (7). For most antibiotics, resistance due to chromosomal mutations is relatively low-level (minimum inhibitory concentration in vitro of 1:~8 $\mu g\ ml^{-1}$), but practical therapeutic problems are posed by such levels of resistance (59).

Penicillin resistance Using transformation as the principal tool, several groups have shown that low-level, non-ß-lactamase resistance of clinical isolates to penicillin (Pen) is due to the additive effects of mutations at several loci (37, 89–91, 154). Although different clinical isolates have been studied genetically by different laboratories, results in general have been remarkably similar (37, 89, 154). Some of these mutations result in resistance only to Pen or other ß-lactam antibiotics, whereas others have pleiotropic effects and result in resistance to multiple compounds (89, 91, 154).

Collectively, mutations at these loci increase the minimum inhibitory concentration (MIC) for Pen from about 0.01 $\mu g\ ml^{-1}$ to about 0.5–1.0 $\mu g\ ml^{-1}$ (37, 91, 154). There probably are other mutations affecting sensitivity to Pen that have not been characterized yet, since clinical isolates with an MIC (Pen G) of 2.0–4.0 μg Pen are not rare (144); unfortunately, it has not been possible yet to construct isogenic strains that express such relatively high levels of chromosomal resistance to Pen.

Mutations at *penA* result in four- to eight-fold increases in resistance to ß-lactam antibiotics only (37, 91, 154), apparently due to reduced binding affinity of penicillin-binding protein (PBP) 2 for Pen (4, 37). There is some evidence that the *penA2* mutation decreases the extent of O-acetylation of peptidoglycan, which may affect susceptibility to lysozyme (168) and, possibly, the ability of peptidoglycan fragments to activate the complement pathways (120). The locus designated *pen* by Maier et al (91) is probably identical to the locus designated *penA* by Sparling et al (154). A second locus designated *pem* is closely linked (90%) to *pen* by transformation and has the phenotypic

effect of increasing about two-fold levels of resistance due to the *pen* mutation (179). The biochemical basis for this modifying effect of *pem* is unknown.

Loci that result in nonspecific low-level resistance to a wide variety of agents have been identified in many gonococci (89, 91, 154). Mutations at *mtr (multiple transformable resistance)* result in partial resistance to many antibiotics, including Pen, tetracycline (Tet), erythromycin (Ery), chloramphenicol (Chl), rifampin (Rif), fusidic acid (Fus), and others, increased resistance to several dyes, and resistance to a variety of detergents and fatty acids (89, 154). The effects of these mutations on resistance to fecal lipids may explain why strains with *mtr* mutations are more common in rectal isolates and in homosexual males (107). The *mtr* mutations result in increased amounts of a 52K outer membrane protein (50), possibly due to a still undetected change in cell envelope chemistry. The physiological effects of *mtr* mutations are to reduce cellular uptake of dyes (and presumably other compounds), resulting in multiple-drug resistance (49).

The genotypically identical *mtr* mutations studied by Maier et al (89) and Sparling et al (154) are not linked to any other known genetic loci in the gonococcus. Another locus studied by Bygdeman et al (21) has an identical phenotype but is almost certainly genetically distinct, because it is very closely linked to a locus affecting the apparent MW and antigenicity of outer membrane Protein I, and does not alter the amounts of the 52K protein. Although Bygdeman et al also designated this locus *mtr* (21), it might be preferable to designate it *nsr (nonspecific resistance)* until its relationship to previously characterized *mtr* mutation is clarified.

Mutations studied by two groups and separately designated *penB* (154) or *tem* (179) have similar phenotypes and genetic map positions. Each results in low-level increases in resistance to Pen and Tet, apparently due to modifying the effects of other mutations (154, 179). The *penB* mutation is very closely linked (98% cotransformation) to loci designated *nmp* that affect the apparent MW and serotype of outer membrane Protein I (25). The biochemical basis for the *penB* phenotype is unknown. The appropriate experiments have not been done to determine whether *penB* and *tem* are the same or distinct loci, but *tem* is known to be linked to the *tet* locus (179), and thus is physically proximate to *penB* (Figure 1).

Linked ribosomal genes Although there is little information about the genetic map of the gonococcus, a significant linkage group involving many of the genes for antibiotic resistance and certain genes of pathogenetic significance is known (90, 135, 149) (Fig. 1). Loci for high-level resistance to streptomycin *(str)* and spectinomycin *(spc)* are known to affect 30S ribosomal response to these drugs (92). Loci for low-level resistance to Fus *(fus)*, Tet *(tet)*, and Chl *(chl)* map between *str* and *spc*. Representatives of each of these loci were from

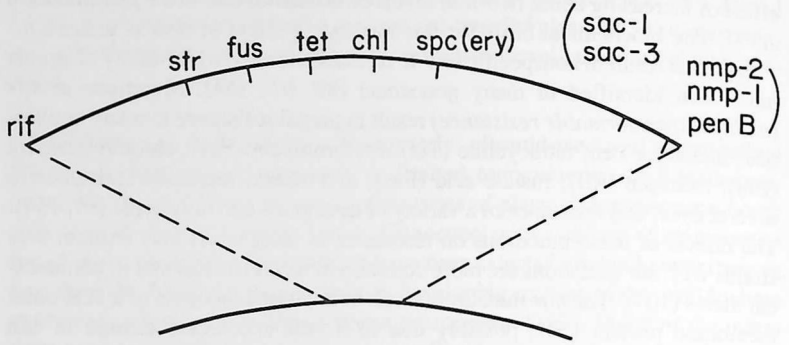


Figure 1 Linked chromosomal genes for antibiotic resistance and cell surface structure. All loci have been mapped by three-factor transformation crosses. Parentheses indicate uncertainties regarding positions of closely linked markers. Other loci whose map positions are less certain but are in or near this cluster include *tem* (modifier for resistance to Pen and Tet located near *tet*, as described in text) and several loci involved in arginine or uracil biosynthesis (F. Jones, personal communication). Symbols: *rif* = rifampin; *str* = streptomycin; *fus* = fusidic acid; *tet* = tetracycline; *chl* = chloramphenicol; *spc* = spectinomycin; *ery* = erythromycin; *sac* = resistance to normal human serum; *nmp* = new membrane protein P.I; *penB* = nonspecific resistance to Pen, Tet, Chl.

clinical isolates, leading to the conclusion that high-level resistance of gonococci to Str and Spc is due to ribosomal mutations. A locus for resistance to Rif *(rif)* is located (in this convention) to the left of this cluster, and *penB* and *nmp* to the right. Cotransformation frequencies are about 10% for *rif* and *str*, 10–20% for *str* and *spc*, and 10–20% for *spc* and *penB* (135, 154).

By separating different sizes of transforming DNA on sucrose gradients, Sox determined that the critical size of DNA required for cotransformation of *rif* and *spc* was about 17×10^6 daltons, whereas DNA of about 10×10^6 daltons was required for cotransformation of *str* and *spc* (149). By extrapolation from these data, the entire cluster from *rif* to *penB* is probably about 30×10^6 daltons, or 3% of the gonococcal genome (76).

Mutations to antibiotic sensitivity Another class of chromosomal mutations designated *env* results in decreased antibiotic resistance, or increased antibiotic sensitivity (93, 137). As many as 15% of random clinical isolates may contain an *env* mutation (40). Introduction of an *env* mutation into a strain containing *penA*, *mtr*, and *penB* results in complete phenotypic suppression of the effects of *mtr* and *penB* (137). There are three genetically distinct but phenotypically identical classes of *env* mutation, each of which recombines with high frequency with the others (recombination index $\gtrsim 1.0$) (40, 137). The *env* mutations are unlinked to other known loci affecting drug resistance, or to other studied

loci (137). Lysko & Morse presented evidence that the effects of *env* mutations on increased sensitivity to multiple drugs, particularly to hydrophobic compounds, is due to increased exposure of outer membrane phospholipids (88).

Almost all clinical isolates with an *env* mutation also contain a phenotypically suppressed *mtr* mutation (40). One explanation for this is provided by the observation that *mtr env* strains grow slightly more rapidly in the absence of antibiotics than isogenic *mtr* derivatives (40); in the presence of antibiotics the *mtr* strain is favored, of course, because the *mtr env* strain is quite antibiotic-sensitive (40, 137). Another piece of evidence that suggests biological disadvantage due to certain mutations to antibiotic resistance is the well-documented rarity of bacteremia with gonococci containing chromosomal mutations to antibiotic resistance (181). (Pc^r strains are not so limited.)

What explanation might there be for this observation? A serum-resistant isolate was not made more sensitive to normal human serum by introduction of *penA*, *mtr*, or *penB* mutations by transformation (31). However, both *penA* and *mtr* increase sensitivity of isogenic strains to nonoxidative killing by a mixture of neutrophil azurophilic granule proteins in vitro (31), suggesting that neutrophils might be more effective in defense against certain antibiotic-resistant gonococci than their antibiotic-sensitive brethren. Shafer recently confirmed and extended these observations by showing that the *penA* and *mtr* mutations result in markedly increased sensitivity in vitro to killing by particular purified azurophilic granule proteins (W. Shafer, personal communication).

The Genetics of Cell Surface Components Affecting Pathogenicity

SERUM RESISTANCE Three genetic loci designated *sac* (resistance to *serum antibody complement*) have been identified (26, 143, 156). Resistance due to the *sac* loci is phenotypically stable on serial subculture. All *sac* loci are linked to the ribosomal *str-spc* gene cluster (26, 143, 156), and two of them *(sac-1, sac-3)* are very closely linked to each other and to the *nmp* loci (Fig. 1). Most of the available genetic evidence does not support a primary role of Protein I in determining resistance to NHS (26, 57, 143), although there is a strong epidemiological association between serum resistance and a particular immunotype of Protein I (24, 139). In isogenic strains, the $sac\text{-}3^+$ locus (sensitivity to NHS) requires presence of considerably higher concentrations of NHS to effect significant killing than does $sac\text{-}1^+$ (143). The gene products of the *sac* loci are unknown; they do not measurably affect the structures of lipopolysaccharide (LPS) or outer membrane proteins (26, 143).

Recently, there have been a number of elegant studies of the immunochemistry of serum resistance in gonococci. Each of these studies employed unrelated serum-sensitive or serum-resistant clinical isolates. In some cases, this has not led to problems of discrepant results—for example, there is a consensus that

serum resistant gonococci are able to survive binding to the cell surface of the terminal complement attack complex (52, 71). In other cases where there is a discrepancy—e.g. the existence of noncomplement fixing antibodies that block the effects of otherwise bactericidal antibodies (100, 123, 138)—it is not clear whether the discrepancies are due to use of different strains, sera, or both. Hopefully, future studies of the immunochemistry of serum resistance will utilize isogenic strains.

Many genotypically serum-sensitive gonococci isolated from patients with uncomplicated disease can be rendered phenotypically serum-resistant after brief exposure to a low-molecular-weight component of human serum (94). Nothing is known of the genetics and little of the immunochemistry or clinical significance of this type of serum resistance.

LIPOPOLYSACCHARIDE LPS is probably involved in pathogenesis of gonococcal infection. Toxic activity of gonococci for organ explants of human fallopian tubes is due principally to the effects of LPS (103); the active moiety probably is the lipid A portion of LPS (47). LPS also appears to be an important target antigen for human antigonococcal bactericidal antibodies (43, 138).

The chemical structure of gonococcal LPS appears similar to rough LPS from *E. coli* and *Salmonella* (50, 119, 183). There are chemical and antigenic differences in the core LPS polysaccharide between strains (2, 119, 183), but little convincing evidence for repeating O antigens (50, 119, 183). LPS structure may be subject to phenotypic variation, depending on culture conditions (108). The biological implications of phenotypic variation in core LPS polysaccharide structure are uncertain but could be important, because variations in growth rates brought about by limitations in availability of utilizable carbon sources resulted in phenotypic conversion from serum resistance to serum sensitivity of one well-studied strain (108).

By use of certain pyocins that recognize core LPS sugar residues as receptors, it is possible to select a variety of pyocin resistant mutants with alterations in LPS (48, 106, 130). For instance, loss of a terminal galactose on the core LPS polysaccharide has no effect on sensitivity to normal human serum, but other mutants with more considerable loss of core polysaccharides are highly sensitive to normal human serum (48). Deep-rough pyocin-resistant mutants with core polysaccharides containing only lipid A and 2-keto-3-deoxyoctonic acid (KDO) are able to activate the classical complement pathway in the apparent absence of antibody (142). Use of pyocins to select LPS mutants, when coupled with monoclonal antibodies specific for epitopes in LPS polysaccharides (1), should allow much better understanding of the structure and function of gonococcal LPS in the near future.

PILI When gonococci are freshly isolated from a patient, the majority of colonies are small and shiny, although some colonies are larger and dull-surfaced (67, 74). The small and shiny colonies, which have been designated types 1 or 2, are virulent; the larger colonies (types 3 and 4) are avirulent (67, 74). Type 1 and 2 colonies contain organisms that produce pili, which are proteinaceous appendages that project from the gonococcal surface (15, 167). The type 1 or 2 phenotype, and the ability to produce pili, is readily lost upon nonselective subculture in the laboratory. A population of type 3 or 4 colonies can give rise to occasional piliated (Pil^+) variants, but at a much lower frequency than the Pil^+ to Pil^- transition. A population composed predominantly of piliated gonococci can be maintained in the laboratory by selective passage of colonies with the appropriate phenotype.

Pili mediate attachment of gonococci to epithelial cell surfaces (20, 64, 68, 160, 180) and play a role in preventing phagocytosis by human polymorphonuclear leukocytes (14, 35). As discussed previously, organisms that are piliated are also competent for genetic transformation (9). Although all gonococcal strains are capable of producing pili, the structure and antigenicity of these components vary from strain to strain. Pili isolated from different strains differ in amino acid composition and subunit molecular weight and show extensive antigenic heterogeneity (18, 56, 117, 128, 140).

Functional and antigenic domains of the pilus subunit for different strains have been studied by cleavage of pilin into three fragments with cyanogen bromide. The N-terminal portion of the molecule (CnBr fragments 1 and 2) is highly conserved and immunorecessive. Binding to red blood cells is mediated by CnBr-2. The C-terminal portion of the molecule (CnBr-3) is a highly variable type-specific determinant that is immunodominant (141).

In addition to the antigenic diversity of pili in different strains, there also is variation in the pilin species expressed by different variants of a single gonococcal strain (85, 132). Heckels' group has isolated four variants of one strain that produce different pilus types, with subunit molecular weights varying from 18,000 to 21,000. The four different types are antigenically distinct, and they differ in efficiency of adherence to different types of host tissues (82, 86, 176, 177).

Peptide mapping studies have shown that the different pilin species share many common peptides, as well as some that are unique to each individual species (82). Swanson (personal communication) has observed that a single gonococcal strain may be capable of producing as many as 12 different pilin species. The ability of the gonococcus to produce antigenically distinct pilin species may be important during infection, as the efficiency of host response to pilin antigens may be circumvented by switching to a different pilin species (55).

A genetic system for the regulation of pilus production in the gonococcus must include mechanisms for the regulation of the high-frequency turning on and off of pilin synthesis, as well as for the generation and regulation of the multiple pilin species that can be made by a single strain. Recent experiments

have revealed one possible mechanism of control of this process. Meyer et al (104) reported the cloning of a structural gene for gonococcal pilin. The conversion from a Pil^+ to Pil^- state was accompanied by rearrangements in the chromosomal sequences that hybridize to the cloned pilin gene. Regulation of gene expression by movable genetic elements has been shown to occur in several different systems, both prokaryotic (146, 173, 185) and eukaryotic (172, 182). The exact relationship between the observed genomic rearrangements and pilus expression control remains to be determined. Recent evidence located two pilus structural genes in a region of about 20 Kb. Transition from Pil^+ to Pil^- was accompanied by deletions of the pilus genes (M. So and T. Meyer, personal communication). Reversion from Pil^- to Pil^+ may be due to genetic exchange between the deleted pilus genes and nearby sequences homologous with the pilus genes and their flanking sequences (M. So, personal communication).

PROTEIN II The existence of another gonococcal surface protein that varies in its expression within a single strain is also revealed by differences in colony morphology. All gonococcal strains form colonies that differ in degree of apparent coloration, or photo-opacity (161, 178). When the outer membrane proteins of these variants are compared by SDS-PAGE, the dark, or opaque colonies often possess one or more surface proteins that are not seen in the corresponding transparent colony variants (53, 83, 162, 164).

These proteins, designated Protein(s) II (P.II), comprise a family of heat-modifiable proteins of apparent molecular weight of 26–30K. A variety of electrophoretically distinct P.II proteins may be found within different colony variants of a single gonococcal strain; some strains have been shown to be capable of expressing six different species of P.II (53). Ordinarily, only one or two of these species of P.II are expressed at one time.

The correspondence between colony opacity and possession of P.II proteins is not complete, because not all P.II proteins contribute to the photo-opacity of gonococcal colonies (110, 165). There has been no direct measurement of the rate of P.II switching, but a switching frequency for colonial opacity of 10^{-3}/cell/generation has been reported (96). Thus, within any gonococcal population of more than a thousand cells, there exists a minority population that differs in expression of P.II proteins.

There is indirect evidence that possession of different P.II proteins may be of significance in different stages of gonococcal infection. Differences in colony opacity of clinical isolates correlate with differences in site of isolation or type of infection (38, 61, 62). There are also differences in virulence of opacity variants in animal models of infection (98, 133, 175). In general, gonococci that form transparent colonies, and that do not possess P.II in the outer membrane, are believed to be more virulent or invasive. Variants with differences in P.II sometimes show differences in serum resistance (63, 84), but

studies of this type have given contradictory results. Expression of P.II affects adherence to various types of host cells, including leukocytes (84).

All of these studies have been complicated by the tendency of P.II-bearing gonococci to form large clumps of organisms, making quantification difficult. Also, with the exception of the excellent studies of Heckels, Lambden, and coworkers that correlate the possession of well-characterized P.II species with different biological properties of gonococci, little of the work on pathobiological significance of P.II has been done with variants that were characterized as to which of the different P.IIs were expressed. Thus, it is difficult to compare the work that has been done in different laboratories, or to draw conclusions about the functional role of individual P.II species of a gonococcal strain.

During the course of a single infection, selective advantage may be conferred by different P.II species at different stages in the infection. It has been suggested that changes in the level of proteases (13, 161), steroid hormones (131), or antibody against P.II (32, 166) may be important in selecting out populations of gonococci that bear different P.II species. In the latter case, the antibody response of the host to P.II proteins might actually be important in selecting for the survival and multiplication of a population of gonococci-lacking P.IIs that might be more invasive and virulent.

Little is known about regulatory mechanisms for P.II expression, although there are several observations pertinent to consideration of models for regulation of P.II. The switching of P.II variants seems to be a phase variation event, because it is reversible, complete, and occurs at a high frequency. Mayer (96) screened almost 200 compounds for their effect on P.II transition rates, but found none with any effect. A single gonococcal cell can, and frequently does, express more than one P.II species at one time, with the loss or acquisition of P.II proteins by gonococci occurring as stepwise events; that is, variants gain or lose P.II proteins one at a time. Swanson has come to this conclusion by observation of colony variants that differ in colony opacity from the majority population (165); a similar conclusion was reached in experiments that examined the P.II profiles of survivors of killing by a bactericidal P.II-specific monoclonal antibody (R. S. Schwalbe & J. G. Cannon, unpublished data).

It is not known if the different P.II species made by a single strain are cleaved or modified forms of a single P.II polypeptide, or if they are products of different structural genes. Peptide mapping studies have shown that different P.IIs of one strain have some unique peptides and many common peptides (54, 163). The results could be consistent with either a single-gene or multi-gene model for P.II synthesis. More complete structural analysis of the proteins should provide information that will help in distinguishing between these possibilities.

Several gonococcal strains that lack the 2.6-Mdal cryptic plasmid show a ten-fold reduction in rate of transition between colony opacity types and presumably between P.II species (96). However, in studies of a pair of strains

differing only in possession or absence of the cryptic plasmid, there was no difference in the frequency of switching of a particular P.II protein (G. D. Biswas, R. S. Schwalbe & P. F. Sparling, unpublished data).

It has been suggested that the regulation of P.II variation might be somehow coordinately regulated with pilus variation, as colony variants that differ in opacity often show differences in both pilus and P.II electrophoretic mobility on SDS-PAGE (132). However, it has been shown that changes in either protein can occur independently of the other one (165).

Preliminary analysis of a cloned gene for P.II from strain FA1090 suggests that structural alterations of DNA accompany changes from expression of one P.II protein to a different one, or from one P.II protein to no P.II protein expressed (W. J. Black & J. G. Cannon, unpublished data). There is no information as to the precise role of such rearrangements in regulation of P.II gene expression. Meyer & So have cloned a structural gene for P.II from strain MS11 and find that major genomic rearrangements do not occur coincident with P.II variations (T. Meyer & M. So, submitted for publication). Clearly, much work needs to be done concerning the genetics of P.II (and of pili), but it seems likely that the structure and function of these proteins and their regulation will be better understood in the near future.

PROTEIN I Protein I (P.I) is the predominant protein species of the gonococcal outer membrane and appears to consist of a single polypeptide chain (70). P.I functions as a porin (12), and has been shown to vary among different gonococcal strains, both in primary structure and apparent molecular weight (70), and in antigenicity. The antigenic differences in different strains form the basis for several different serotyping systems for gonococci (69, 134, 169). An interesting recent observation was made by Blake & Gotschlich, who showed that P.I may insert into human red blood cell membranes (12). It is possible (but unproved) that a similar insertion may promote internalization of gonococci by epithelial cells (12).

Epidemiological studies have revealed interesting associations between particular P.I serotypes and certain other properties of the gonococcus, including propensity to cause bacteremia, antibiotic sensitivity, and serum resistance (19, 24). As mentioned above, most genetic studies have shown that these properties are genetically separable from the P.I phenotype (25, 26).

Genetic loci affecting P.I have been designated *nmp* loci (for *n*ew *m*embrane *p*rotein) (25, 26). In transformation experiments in which donor and recipient strains differ in P.I, the donor P.I replaces the recipient P.I as a consequence of introduction of the *nmp* locus. The *nmp* loci are linked by transformation to the antibiotic resistance markers shown on the genetic map in Fig. 1 (25, 26). It is not known if the *nmp* loci are the structural gene(s) for P.I, or whether they act on P.I in some other way.

Introduction of *nmp-1* results in expression of a new P.I that has quite different primary structure than the recipient's original P.I (25), suggesting that the two species of P.I might be products of different structural genes. Recently, Guymon isolated spontaneous mutants that showed changes in apparent molecular weight and immunochemical type of P.I (personal communication). It is possible that the gonococcal genome contains more than one gene for P.I similar to the multiple porin genes that have been described in other organisms, and that only one of the genes is expressed. If there is variation in P.I expression within a strain, it probably occurs at a frequency much lower than that observed for pili and P.II.

IRON-REGULATED PROTEINS (FeRPs) Gonococci express markedly increased amounts of a number of outer membrane proteins under conditions of iron starvation in vitro (115). There are strain variations in these proteins, but the most common FeRPs have apparent M_r in SDS-PAGE of about 100K, 70K, and 37K (115; T. Meitzner & S. Morse, personal communication; S. West & P. F. Sparling, unpublished data). These proteins are analogous to iron-repressible proteins in many other bacteria, some of which are known to be receptors involved in iron uptake. There is conflicting evidence as to whether gonococci make siderophores (116, 184). It is possible that gonococci utilize outer membrane FeRPs to help acquire essential iron in vivo in the absence of siderophores, as postulated for meningococci by Simonson et al (147).

Genetic approaches to the relationship between FeRPs and the ability of gonococci to acquire essential iron from serum transferrin, mucosal lactoferrin, or hemin are just commencing. Ability to use 25% iron-saturated lactoferrin (LF) as an iron source is correlated with ability of certain strains to cause symptomatic urethritis or salpingitis (105). Nonlactoferrin utilizing strains (LF^-) are readily transformed to lactoferrin-utilizers (LF^+) by transforming DNA from LF^+ donors (unpublished data). The naturally occurring LF^- mutation apparently affects a surface receptor for binding LF (T. Meitzner & S. Morse, personal communication).

SURFACE PROTEINS COMMON TO ALL GONOCOCCI In addition to the highly variable surface proteins that have been discussed, there are also some components that appear to be common to all gonococci. These include Protein III, an outer membrane protein of apparent molecular weight of 30K, that appears to be identical by peptide mapping in all strains that have been studied (72, 101, 111). Also, Newhall et al have described a high-molecular-weight complex designated OMP-MC, found in the outer membrane of all gonococci (112). No genetic analysis has been applied to these components to date.

Cannon et al (23) have identified an additional common antigen, designated the H.8 antigen, that is present on all gonococci and meningococci tested, but

not on most commensal *Neisseria*. A monoclonal antibody specific for the H.8 antigen binds to a surface-exposed outer membrane protein with an apparent molecular weight of 20K when solubilized at 37° C, and of 30K when solubilized at 100° C. The gene for this component has been cloned in bacteriophage lambda. The commensal *Neisseria* that do not express the H.8 antigen do not show hybridization of genomic DNA to the cloned gonococcal gene (11). Further investigations of the genetics and function of the H.8 protein should be facilitated by the availability of the cloned gene.

IgA PROTEASE Gonococci, among other mucosal pathogens, produce an extracellular protease that specifically cleaves human IgA1 (109, 122). It has been postulated that this enzyme might aid the organisms in avoiding IgA-mediated host defenses at the mucosal surface. However, because all gonococcal strains that have been examined produce the enzyme, it has been impossible to directly test the role of this enzyme in gonococcal pathogenesis. Koomey et al (80) cloned the gene for IgA1 protease, and showed that the commensal *Neisseria,* which do not make IgA1 protease, do not contain DNA sequences homologous to the cloned gonococcal gene.

IgA1 protease-deficient mutants of *N. gonorrhoeae* were isolated by reintroduction of deletions of the cloned gene into the gonococcal genome by transformation. In this manner, gonococcal strains differing only in the ability to produce this enzyme were constructed. However, the substrate specificity of this enzyme for human IgA1 imposes limitations on the systems that can be used to study the effect of the enzyme on gonococcal virulence. This work illustrates both the power of recombinant DNA technology to aid in investigations of pathogenesis, and also the predicament facing the investigator who wishes to apply this technology to an organism such as the gonococcus that is an obligate human pathogen.

SUMMARY AND CONCLUDING REMARKS

Despite the inherent limitations imposed by working with an organism that still has a very limited genetic map and relatively few systems for manipulation of the genome, much has been learned in the past decade. Recent applications of the technology of monoclonal antibodies and recombinant DNA, coupled with careful studies of the immunobiology of pili and various outer membrane structures have provided exciting insights into the molecular pathogenesis of gonococcal infections. One of the principal lessons is the sophistication of the gonococcal strategies for evading the host defenses, including high-frequency variations of pili and P.II and an extracellular protease for specific cleavage of IgA1. It is unclear how much antigenic variation will limit vaccine development. Regardless, the pursuit of a vaccine has led to important new fundamental knowledge of the genetics and structure-function relationships of several cell surface components, and the future promises to be both rational and interesting.

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Literature Cited

1. Apicella, M. A., Bennett, K. M., Hermerath, C.A., Roberts, D. E. 1981. *Infect. Immun.* 34:751–56
2. Apicella, M. A., Gagliardi, N. C. 1979. *Infect. Immun.* 26:870–74
3. Ashford, W. A., Golash, R. G., Hemming, V. G. 1976. *Lancet* 2:657–58
4. Barbour, A. G. 1981. *Antimicrob. Agents Chemother.* 19:316–22
5. Bergstrom, S., Norlander, L., Norqvist, A., Normark, S. 1978. *Antimicrob. Agents Chemother.* 13:618–23
6. Biswas, G. D., Blackman, E. Y., Sparling, P. F. 1980. *J. Bacteriol.* 143:1318–24
7. Biswas, G., Comer, S., Sparling, P. F. 1976. *J. Bacteriol.* 125:1207–10
8. Biswas, G. D., Graves, J. F., Sox, T. E., Tenover, F. C., Sparling, P. F. 1982. *J. Bacteriol.* 151:77–82
9. Biswas, G. D., Sox, T., Blackman, E., Sparling, P. F. 1977. *J. Bacteriol.* 129:983–92
10. Biswas, G. D., Sparling, P. F. 1981. *J. Bacteriol.* 145:638–40
11. Black, W. J., Cannon, J. G. 1983. *Abstr. D6,* Am. Soc. Microbiol.
12. Blake, M. S., Gotschlich, E. C. 1983. *Prog. Allergy* 33:298–313
13. Blake, M. S., Gotschlich, E. C., Swanson, J. 1981. *Infect. Immun.* 33:212–22
14. Blake, M. S., Swanson, J. 1975. *Infect. Immun.* 11:1402–4
15. Brinton, C. C., Bryan, J., Dillon, J. A., Guerina, N., Jacobson, L. J., et al. 1978. See Ref. 15a, pp. 155–78
15a. Brooks, G. F., Gotschlich, E. C., Holmes, K. K., Saunders, W. D., Young, F. E., eds. 1978. *Immunobiology of Neisseria Gonorrhoeae.* Washington DC: Am. Soc. Microbiol. 400 pp.
16. Brunton, J., Bennett, P., Grinsted, J. 1981. *J. Bacteriol.* 148:788–95
17. Brunton, J., Meier, M., Ehrman, N., Maclean, I., Slaney, L., et al. 1982. *Antimicrob. Agents Chemother.* 21:857–63
18. Buchanan, T. M. 1975. *J. Exp. Med.* 141:1470–75
19. Buchanan, T. M., Hildebrandt, J. F. 1981. *Infect. Immun.* 32:985–94
20. Buchanan, T. M., Pearce, W. A. 1976. *Infect. Immun.* 13:1483–89
21. Bygdeman, S., Backman, M., Danielsson, D., Norgren, M. 1982. *Acta Path. Microbiol. Immunol. Scand. Sect. B* 90:243–50
22. Campbell, L. A., Yasbin, R. E. 1979. *J. Bacteriol.* 140:1109–11
23. Cannon, J. G., Black, W. J., Nachamkin, I., Stewart, P. 1984. *Infect. Immun.* 43:994–99
24. Cannon, J. G., Buchanan, T. M., Sparling, P. F. 1983. *Infect. Immun.* 40:816–19
25. Cannon, J. G., Klapper, D. G., Blackman, E. Y., Sparling, P. F. 1980. *J. Bacteriol.* 143:847–51
26. Cannon, J. G., Lee, T. J., Guymon, L. F., Sparling, P. F. 1981. *Infect. Immun.* 32:547–52
27. Catlin, B. W. 1973. *J. Infect. Dis.* 128: 178–94
28. Catlin, B. W. 1974. *J. Bacteriol.* 120: 203–9
29. Catlin, B. W. 1976. *Microbiology—1976,* ed. D. Schlessinger, pp. 453–66. Washington DC: Am. Soc. Microbiol.
30. Contente, S., Dubnau, D. 1979. *Plasmid* 2:555–71
31. Daly, J. A., Lee, T. J., Spitznagel, J. K., Sparling, P. F. 1982. *Infect. Immun.* 35:826–33
32. Diaz, J. L., Heckels, J. E. 1982. *J. Gen. Microbiol.* 128:585–91
33. Dickgiesser, N., Bennett, P. M., Richmond, M. H. 1982. *J. Bacteriol.* 151: 1171–5
34. Dillon, J. R., Pauze, M., Yeung, K.-H. 1983. *Lancet* 1:797–81

35. Dilworth, J. A., Hendley, J. O., Mandell, G. C. 1975. *Infect. Immun.* 11:512–16
36. Dougherty, T. J., Asmus, A., Tomasz, A. 1979. *Biochem. Biophys. Res. Commun.* 86:97–104
37. Dougherty, T. J., Koller, A. E., Tomasz, A. 1980. *Antimicrob. Agents Chemother.* 18:730–37
38. Draper, D. L., James, J. F., Brooks, G. F., Sweet, R. L. 1980. *Infect. Immun.* 27:882–88
39. Eisenstein, B. I., Sox, T., Biswas, G., Blackman, E., Sparling, P. F. 1977. *Science* 195:998–1000
40. Eisenstein, B. I., Sparling, P. F. 1978. *Nature* 271:242–44
41. Fayet, O., Froment, Y., Piffaretti, J.-C. 1982. *J. Bacteriol.* 149:136–44
42. Flett, F., Humphreys, G. O., Saunders, J. R. 1981. *J. Gen. Microbiol.* 125:123–29
43. Glynn, A. A., Ward, M. E. 1970. *Infect. Immun.* 2:162–68
44. Goldberg, I. D., Steinberg, V. I., Siddiqui, A., Hart, E. J., Schaper, D. 1978. See Ref. 15a, pp. 55–59
45. Goodgal, S. H., Herriott, R. M. 1961. *J. Gen. Physiol.* 44:1201–27
46. Graves, J. F., Biswas, G. D., Sparling, P. F. 1982. *J. Bacteriol.* 152:1071–77
47. Gregg, C. R., Melly, M. A., Hellerquist, C. G., Coniglio, J. G., McGee, Z. A. 1981. *J. Infect. Dis.* 143:432–39
48. Guymon, L. F., Esser, M., Shafer, W. M. 1982. *Infect. Immun.* 36:541–47
49. Guymon, L. F., Sparling, P. F. 1975. *J. Bacteriol.* 124:757–63
50. Guymon, L. F., Walstad, D. L., Sparling, P. F. 1978. *J. Bacteriol.* 136:391–401
51. Handsfield, H. H., Murphy, V. L. 1983. *Lancet* 2:67–70
52. Harriman, G. R., Podack, E. R., Braude, A. I., Corbeil, L. C., Esser, A. F., et al. 1982. *J. Exp. Med.* 156:1235–49
53. Heckels, J. E. 1977. *J. Gen. Microbiol.* 99:333–41
54. Heckels, J. E. 1981. *J. Bacteriol.* 145: 736–42
55. Heckels, J. 1982. See Ref. 137a, pp. 301–4
56. Hermodson, M. A., Chen, K. C. S., Buchanan, T. M. 1978. *Biochemistry* 17:442–45
57. Hildebrandt, J. F., Mayer, L. W., Wang, S. P., Buchanan, T. M. 1978. *Infect. Immun.* 20:267–73
58. Ison, C., Glynn, A. A., Bascomb, S. 1982. *J. Clin. Pathol.* 35:1153–57
59. Jaffe, H. W., Biddle, J. W., Thornsberry, C., Johnson, R. E., Kaufman, R. E., et al. 1976. *N. Engl. J. Med.* 294: 5–9
60. Jaffe, H. W., Kraus, S. J., Edwards, T. A., Weinberger, S. S., Zubrzycki, L. 1982. *J. Infect. Dis.* 146:275–79
61. James, J. F., Swanson, J. 1978. *Infect. Immun.* 19:332–40
62. James, J. F., Swanson, J. 1978. See Ref. 15a, pp. 338–43
63. James, J. F., Zurlinden, E., Lammel, C. J., Brooks, G. F. 1982. *J. Infect. Dis.* 145:37–44
64. James-Holmquest, A. N., Swanson, J., Buchanan, T. M., Wende, R. D., Williams, R. P. 1974. *Infect. Immun.* 9:897–902
65. Janik, A., Juni, E., Heym, G. A. 1976. *J. Clin. Microbiol.* 4:71–81
66. Jephcott, A. E., Eggleston, S. I., Copley, C. 1982. *Lancet* 1:1467–68
67. Jephcott, A. E., Reyn, A. 1971. *Acta Pathol. Microbiol. Scand. Sect. B* 79: 609–14
68. Johnson, A. P., Taylor-Robinson, D., McGee, Z. A. 1977. *Infect. Immun.* 18:833–39
69. Johnston, K. H. 1980. *Infect. Immun.* 28:101–10
70. Johnston, K. H., Holmes, K. K., Gotschlich, E. C. 1976. *J. Exp. Med.* 143: 751–58
71. Joiner, K. A., Warren, K. A., Brown, E. J., Swanson, J., Frank, M. M. 1983. *J. Immunol.* 131:1443–51
72. Judd, R. 1982. *Infect. Immun.* 37:622–31
73. Juni, E., Heym, G. A. 1980. *J. Gen. Microbiol.* 121:85–92
74. Kellogg, D. S., Peacock, W. L., Deacon, W. E., Brown, L., Pirkle, C. I. 1963. *J. Bacteriol.* 85:1274–79
75. Kenyon, J. E. 1978. *Current Microbiol.* 1:253–56
76. Kingsbury, D. T. 1969. *J. Bacteriol.* 98:1400–01
77. Kirven, L. A., Thornsberry, C. 1977. *Antimicrob. Agents Chemother.* 11: 1004–6
78. Knapp, J. S., Holmes, K. K. 1975. *J. Infect. Dis.* 132:204–08
79. Kolodkin, A. B., Clark, V. L., Tenover, F. C., Young, F. E. 1982. *Infect. Immun.* 36:586–90
80. Koomey, J. M., Gill, R. E., Falkow, S. 1982. *Proc. Natl. Acad. Sci. USA* 79: 7881–85
81. Korch, C., Hagblom, P., Normark, S. 1983. *J. Bacteriol.* 155:1324–32
82. Lambden, P. R. 1982. *J. Gen. Microbiol.* 128:2105–11
83. Lambden, P. R., Heckels, J. E. 1979. *FEMS Microbiol. Lett.* 5:263–65

84. Lambden, P. R., Heckels, J. E., James, L. T., Watt, P. J. 1979. *J. Gen. Microbiol.* 114:305–12
85. Lambden, P. R., Heckels, J. E., McBride, H., Watt, P. J. 1981. *FEMS Microbiol. Lett.* 10:339–41
86. Lambden, P. R., Robertson, J. N., Watt, P. J. 1980. *J. Bacteriol.* 141:393–96
87. Laufs, R., Kaulfers, P.-M., Jahn, G., Teschner, U. 1979. *J. Gen. Microbiol.* 111:223–31
88. Lysko, P. G., Morse, S. A. 1981. *J. Bacteriol.* 145:946–52
89. Maier, T. W., Warner, P., Zubrzycki, L., Chila, M. 1977. *Antimicrob. Agents Chemother.* 12:444–46
90. Maier, T. W., Zubrzycki, L., Coyle, M. B. 1975. *Antimicrob. Agents Chemother.* 7:676–81
91. Maier, T. W., Zubrzycki, L., Coyle, M. B., Chila, M., Warner, P. 1975. *J. Bacteriol.* 124:834–42
92. Maness, M. J., Foster, G. C., Sparling, P. F. 1974. *J. Bacteriol.* 120:1293–99
93. Maness, M. J., Sparling, P. F. 1973. *J. Infect. Dis.* 128:321–30
94. Martin, P. M. V., Patel, P. V., Parsons, N. J., Smith, H. 1981. *J. Gen. Microbiol.* 127:213–17
95. Mathis, L. S., Scocca, J. J. 1982. *J. Gen. Microbiol.* 128:1159–61
96. Mayer, L. W. 1982. *Infect. Immun.* 37: 481–85
97. Mayer, L. W., Schoolnik, G. K., Falkow, S. 1977. *Infect. Immun.* 18:165–72
98. McBride, H. M., Lambden, P. R., Heckels, J. E., Watt, P. J. 1981. *J. Gen. Microbiol.* 126:63–67
99. McCutchan, J. A., Adler, M. W., Berrie, J. R. H. 1982. *Brit. Med. J.* 285:337–40
100. McCutchan, J. A., Katzenstein, D., Norquist, D., Chikami, G., Wunderlich, A., et al. 1978. *J. Immunol.* 121:1884–88
101. McDade, R. L. Jr., Johnston, K. H. 1980. *J. Bacteriol.* 141:1183–91
102. McNicol, P. J., Albritton, W. L., Ronald, A. R. 1983. *J. Bacteriol.* 156: 437–40
103. Melly, M. A., Gregg, C. R., McGee, Z. A. 1981. *J. Infect. Dis.* 143:423–31
104. Meyer, T. M., Mlawer, N., So, M. 1982. *Cell* 30:45–52
105. Mickelsen, P. A., Blackman, E., Sparling, P. F. 1982. *Infect. Immun.* 35:915–20
106. Morse, S. A., Apicella, M. A. 1982. *J. Infect. Dis.* 145:206–16
107. Morse, S. A., Lysko, P. G., McFarland, L., Knapp, J. S., Sandstrom, E., et al. 1982. *Infect. Immun.* 37:432–38
108. Morse, S. A., Mintz, C. S., Sarafian, S. K., Bartenstein, L., Bertram, M., Apicella, M. A. 1983. *Infect. Immun.* 41:74–82
109. Mulks, M. H., Kornfeld, S. J., Plaut, A. G. 1980. *J. Infect. Dis.* 139:450–56
110. Nachamkin, I., Cannon, J. G., Mittler, R. S. 1981. *Infect. Immun.* 32:641–48
111. Newhall, W. J., Sawyer, W. D., Haak, R. A. 1980. *Infect. Immun.* 28:785–91
112. Newhall, W. J., Wilde, C. E. III, Sawyer, W. D., Haak, R. A. 1980. *Infect. Immun.* 27:475–82
113. Ng, W. W. S., Echeverria, P., Rockhill, R., Duangmani, C. 1982. *Sex. Transm. Dis.* 9:120–23
114. Norlander, L., Davies, J., Normark, S. 1979. *J. Bacteriol.* 138:756–61
115. Norqvist, A., Davies, J., Norlander, L., Normark, S. 1978. *FEMS Microbiol. Lett.* 4:71–75
116. Norrod, P., Williams, R. P. 1978. *Current Microbiol.* 1:281–84
117. Penn, C. W., Parsons, N. J., Veale, D. R., Smith, H. 1980. *J. Gen. Microbiol.* 121:195–202
118. Perine, P. L., Thornsberry, C., Schalla, W., Biddle, J., Siegel, M. S., et al. 1977. *Lancet* 2:993–95
119. Perry, M. B., Daoust, V., Johnson, K. G., Diena, B. B., Ashton, F. E. 1978. See Ref. 15a, pp. 101–7
120. Petersen, B. H., Rosenthal, R. S. 1982. *Infect. Immun.* 35:442–8
121. Phillips, I. 1976. *Lancet* 2:656–57
122. Plaut, A. G., Gilbert, J. V., Artenstein, M. S., Capra, J. D. 1975. *Science* 190:1103–5
123. Rice, P. A., Kasper, D. L. 1982. *J. Clin. Invest.* 70:157–67
124. Roberts, M., Elwell, L. P., Falkow, S. 1977. *J. Bacteriol.* 131:557–63
125. Roberts, M., Falkow, S. 1977. *Nature* 266:630–31
126. Roberts, M., Falkow, S. 1978. *J. Bacteriol.* 134:66–70
127. Roberts, M., Falkow, S. 1979. *Infect. Immun.* 24:982–84
128. Robertson, J. P., Vincent, P., Ward, M. E. 1977. *J. Gen. Microbiol.* 102:169–77
129. Rockhill, R. C., Leksana, B., Wijaya, U., Martosudarmo, D., Soekanto, W., Widejatno, W. 1982. *Southeast Asian J. Trop. Med. Public Health* 13:96–9
130. Sadoff, J. C., Zollinger, W. D., Sidberry, H. 1978. See Ref. 15a, pp. 93–100
131. Salit, I. E. 1982. *Can. J. Biochem.* 60:301–6
132. Salit, I. E., Blake, M., Gotschlich, E. C. 1980. *J. Exp. Med.* 151:716–25
133. Salit, I. E., Gotschlich, E. C. 1978. *Infect. Immun.* 22:359–64
134. Sandstrom, E. G., Chen, K. C. S.,

Buchanan, T. M. 1982. *Infect. Immun.* 38:462–70
135. Sarubbi, F. A. Jr., Blackman, E., Sparling, P. F. 1974. *J. Bacteriol.* 120:1284–92
136. Sarubbi, F. A. Jr., Sparling, P. F. 1974. *J. Infect. Dis.* 130:660–63
137. Sarubbi, F. A. Jr., Sparling, P. F., Blackman, E., Lewis, E. 1975. *J. Bacteriol.* 124:750–56
137a. Schlessinger, D., ed. 1982. *Microbiology—1982.* Washington DC: Am. Soc. Microbiol. 420 pp.
138. Schneider, H., Griffiss, J. M., Williams, G. D., Pier, G. B. 1982. *J. Gen. Microbiol.* 128:13–22
139. Schoolnik, G. K., Buchanan, T. M., Holmes, K. K. 1976. *J. Clin. Invest.* 58:1163–73
140. Schoolnik, G. K., Tai, J. Y., Gotschlich, E. C. 1982. *Bacterial Vaccines,* Vol. 4, ed. J. B. Robbins, J. C. Hill, G. Sadoff, pp. 172–180. New York: Thieme Stratton
141. Schoolnik, G. K., Tai, J. Y., Gotschlich, E. C. 1982. See Ref. 137a, pp. 312–16
142. Shafer, W. M., Joiner, K., Guymon, L. F., Cohen, M. S., Sparling, P. F. 1984. *J. Infect. Dis.* 149:175–83
143. Shafer, W. M., Guymon, L. F., Sparling, P. F. 1982. *Infect. Immun.* 35:764–69
144. Shtibel, R. 1980. *Lancet* 2:39
145. Silver, L. E., Clark, V. L., Stein, D. C. 1983. *Abstr. D75,* Am. Soc. Microbiol.
146. Simon, M., Zieg, J., Silverman, M., Mandel, G., Doolittle, R. 1980. *Science* 209:1370–74
147. Simonson, C., Brener, D., DeVoe, I. W. 1982. *Infect. Immun.* 36:107–13
148. Sisco, K. L., Smith, H. O. 1979. *Proc. Natl. Acad. Sci. USA* 76:972–76
149. Sox, T. E. 1978. *The Genetic Transfer of Antibiotic Resistance in Neisseria Gonorrhoeae.* Ph.D. thesis. Univ. No. Carolina, Chapel Hill. 227 pp.
150. Sox, T. E., Mohammed, W., Blackman, E., Biswas, G., Sparling, P. F. 1978. *J. Bacteriol.* 134:278–86
151. Sox, T. E., Mohammed, W., Sparling, P. F. 1979. *J. Bacteriol.* 138:510–18
152. Sparling, P. F. 1966. *J. Bacteriol.* 92:1364–71
153. Sparling, P. F., Biswas, G., Sox, T. E. 1977. *The Gonococcus,* ed. R. B. Roberts, pp. 155–76. New York: Wiley & Sons
154. Sparling, P. F., Sarubbi, F. A. Jr., Blackman, E. 1975. *J. Bacteriol.* 124: 740–49
155. Sparling, P. F., Sox, T. E., Mohammed, W., Guymon, L. F. 1978. See Ref. 15a, pp. 44–52
156. Spratt, S. K., Jones, F., Shockley, T. E., Jackson, J. H. 1980. *Infect. Immun.* 29:287–89
157. Stein, D. C., Silver, L. E., Clark, V. L., Young, F. E. 1983. *Gene* 25:241–47
158. Stein, D. C., Young, F. E., Tenover, F. C., Clark, V. L. 1983. *Mol. Gen. Genet.* 189:77–84
159. Steinberg, V. I., Goldberg, I. D. 1980. *J. Bacteriol.* 142:350–54
159a. Stuy, J. H. 1965. *J. Mol. Biol.* 13:554–70
160. Swanson, J. 1973. *J. Exp. Med.* 127: 571–89
161. Swanson, J. 1978. *Infect. Immun.* 19: 320–31
162. Swanson, J. 1978. *Infect. Immun.* 21: 292–302
163. Swanson, J. 1980. *Infect. Immun.* 28: 54–64
164. Swanson, J. 1981. *Infect. Immun.* 34: 804–16
165. Swanson, J. 1982. *Infect. Immun.* 37: 359–68
166. Swanson, J., Barrera, O. 1983. *J. Exp. Med.* 157:1405–20
167. Swanson, J., Kraus, S. J., Gotschlich, E. C. 1971. *J. Exp. Med.* 134:886–905
168. Swim, S. C., Gfell, M. A., Wilde, C. E. III, Rosenthal, R. S. 1982. *Infect. Immun.* 42:446–52
169. Tam, M. R., Buchanan, T. M., Sandstrom, E. G., Holmes, K. K., Knapp, J. S., et al. 1982. *Infect. Immun.* 36:1042–53
170. Tenover, F. C., Mayer, L. W., Young, F. E. 1980. *Infect. Immun.* 29:181–85
171. Thornsberry, C., Biddle, J. W., Perine, P. L., Siegel, M. S. 1978. See Ref. 15a, pp. 62–67
172. Tonegawa, S., Sakano, H., Maki, R., Traunecker, H. G., Roeder, W., Kurosawa, Y. 1980. *Cold Spring Harbor Symp. Quant. Biol.* 45:839–58
173. Van de Putte, P., Cramer, S., Giphart-Gassler, M. 1980. *Nature* 286:218–22
174. van Klingeren, B., van Wijngaarden, L. J., Dessens-Kroon, M., van Embden, J. D. 1983. *J. Antimicrob. Chemother.* 11:15–20
175. Virji, M., Everson, J. S. 1981. *Infect. Immun.* 31:965–70
176. Virji, M., Everson, J. S., Lambden, P. R. 1982. *J. Gen. Microbiol.* 128:1095–1100
177. Virgi, M., Heckels, J. E., Watt, P. J. 1983. *J. Gen. Microbiol.* 129:1965–73
178. Walstad, D. L., Guymon, L. F., Sparling, P. F. 1977. *J. Bacteriol.* 129:1623–27
179. Warner, P. F., Zubrzycki, L. J., Chila, M. 1980. *J. Gen. Microbiol.* 117:103–10
180. Watt, P. J., Ward, M. E. 1980. *Bacterial Adherence (Receptors and Recognition,* Series B, Vol. 6), ed. E. H. Beachey, pp. 253–88. London: Chapman & Hall
181. Wiesner, P. J., Handsfield, H. H., Holmes, K. K. 1973. *N. Engl. J. Med.* 288:1221–22
182. Williams, R. O., Young, J. R., Majewa, P. A. O. 1979. *Nature* 282:847–49
183. Wiseman, G. M., Caird, J. D. 1977. *Infect. Immun.* 16:550–56
184. Yancey, R. J., Finkelstein, R. A. 1981. *Infect. Immun.* 32:600–8
185. Zieg, J., Simon, M. 1980. *Proc. Natl. Acad. Sci. USA* 77:4196–200

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NUTRIENT TRANSPORT BY ANOXYGENIC AND OXYGENIC PHOTOSYNTHETIC BACTERIA

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INTRODUCTION

The energy source of photosynthetic organisms is an electromagnetic radiation and thus does not depend on the interaction of cytoplasmic membrane with molecules in the surrounding solution. The use of light energy permits the complete separation of the two functions of nutrient acquisition and ATP synthesis, which in heterotrophs are intimately linked and dependent on move-

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ments of complex nutrients into the cell. This dissociation has both quantitative and qualitative consequences. It permits an organic carbon source to be converted to cell material with higher efficiency than is possible for an aerobic, nonphotosynthetic organism; a versatile organotroph like *Rhodopseudomonas sphaeroides*, growing photosynthetically (under anaerobic conditions) in continuous culture with limiting malate, can produce approximately twice as much protein as is obtained in the same medium if the organism is grown aerobically in darkness (unpublished observations). A comparison of cell yields with a fermentative organism would be even more striking.

Of even greater importance in natural ecosystems, an independent energy source opens up the possibility of using for biosynthesis compounds that are simply not utilizable by other organisms in the environment. Many of the anoxygenic green and purple bacteria contrive to use the most oxidized form of carbon as a major source, forcing CO_2 reduction with electrons derived from the oxidation of reduced sulfur compounds.

As shown many years ago, the extent of fixation depends on light energy, not on the oxidative energy yield (73). The most favored carbon sources for the photoheterotrophic groups of organisms tend to be fatty or dicarboxylic acids, the end products of fermentative processes, rather than the sugars that would bring them into competition with the fermenters themselves. This happens because the availability of an independent energy source makes it possible for the organisms to adjust the state of reduction of the substrate to the needs of biosynthesis by the simple device of adding or eliminating CO_2. The oxygenic cyanobacteria, of course, have the simplest nutrient requirements of all. A further consequence of ability to use light energy, whose flux at intervals, at least, is likely to exceed the demands of biosynthesis, is the wide distribution of the capacity for the highly energy-demanding process of dinitrogen reduction. Photosynthetic bacteria thus gain access to a generally plentiful source of an essential cell constituent that is available to only a small number of nonphotosynthetic competitors.

The overall macromolecular composition of photosynthetic microorganisms differs little from that of heterotrophs. From rather fragmentary information, the intracellular environment, in terms of ionic, nucleotide, and other metabolite composition, appears to be very similar in all bacteria. The fundamental properties of phospholipid membranes, in the sense of relatively high permeability to gases and impermeability to polar or charged constituents (42, 119), seems common to all biological systems. It may therefore be expected that the molecular mechanisms of nutrient uptake systems of photosynthetic organisms should have many features in common with those extensively investigated in heterotrophs. The wider choices of carbon and nitrogen source available to photosynthetic organisms will affect the chemical nature of the nutrients; for other essential cellular constituents such as phosphorus, the photosynthetic organism has no wider options than any other.

The many investigations of nutrient uptake in photosynthetic bacteria have had extraordinarily different focuses. The ecologist has been primarily concerned with the competitive effectiveness of these processes, in comparison to those of other organisms. At the other end of the scale, the rapid progress in understanding of light energy conversion, and the unique manipulability of light as an energy source, have prompted investigations in which details of mechanism have been emphasized. In some investigations, therefore, the process of transport across the cytoplasmic membrane has not been separated from subsequent metabolism, so that it may not be clear whether transport is active or facilitated. The whole process studied should properly be termed uptake, rather than transport. Studies of the transport mechanism itself have tended to use analogs of the true substrate, or membrane vesicles, which makes it hard to assess the functional adequacy of the system in the economy of the growing cell. The arrangement adopted in this article involves examination of the movement of nutrients across the cytoplasmic membranes of both classical anoxygenic and oxygenic photosynthetic organisms, grouped according to the elements they supply. It excludes Halobacteria. This organization has the advantage of making comparison between the physiological types of organisms relatively easy, but it may tend to obscure the extensive interactions that must occur between the transport of different compounds, particularly where secondary active transport is involved.

Balanced growth of any organism depends on availability of all macromolecular monomers, and, although the ability of photosynthetic organisms to take in and store an excess of one nutrient in the absence of others is well developed, the instantaneous interaction of transport systems in a growing cell must be very extensive, if at present little explored.

MEMBRANE TRANSPORT: GENERAL

Living cells contain organic metabolites at concentrations generally of the order of 0.05–20 mM, certainly well above what is likely to be encountered in most of the environments where photosynthetic bacteria are found. Organisms that are able to manufacture all of their biosynthetic intermediates from the simplest gaseous ingredients like CO_2, H_2, and N_2—and this includes a great number of the photosynthetic bacteria—might conceivably be able to maintain high concentration gradients by simple impermeability of the cytoplasmic membrane.

However, other essential inorganic nutrients such as phosphate, mono- and divalent cations, and anions are clearly required to cross the cytoplasmic membrane. Experimentation with a wide range of microorganisms has indicated the need for, and existence of, both facilitated diffusion and active transport systems for selective uptake of charged or highly polar nutrients. Provided that the internal pool can be kept low by chemical modification of entered substrate, facilitated or even simple diffusion may be a very effective

method of acquiring needed nutrients from very dilute environments. However, all organisms that have been studied in any detail possess in addition a great many systems that can achieve higher concentrations of their substrates inside the cell than outside.

Photosynthetic organisms presumably face strong competition from each other and from different physiological types for most or all of the molecules that are needed as starting points for their biosyntheses. Highly efficient uptake systems may therefore be expected. It is not possible, or indeed proper, to give more than a very brief sketch of current views on membrane transport in free-living cells, and a great many reviews concerned with different aspects have appeared in the last few years. (Particularly useful are 30, 42–44, 71, 119, 155).

The concept of a central role of proton motive force as originally developed by Mitchell (92) is generally accepted, and active transport is seen as being of two kinds: primary, following on membrane electron transport or ATP hydrolysis and involving changes in covalent bonds; and secondary, in which the primary ion gradient is used to bring about the movement of other components against their concentration gradients. The total proton motive force Δp, expressed in mV, has two components: Ψ, the membrane potential generated by the effective movement of H^+ from one side of the membrane to the other, and a term related to the pH difference, so that

$$\Delta p = \Delta\tilde{\mu}_H+/F = \Psi - (2.3RT/F)\,\Delta pH,$$

where F is the Faraday, and R and T have their usual meaning; 2.3RT/F is close to 60 mV at about 25°C.

The methods available for measuring these components have been assessed critically (4, 29, 30, 79, 87, 126). Values of Δp of about 120–130 mV are commonly found, in active photosynthetic as well as nonphotosynthetic organisms (e.g. 18, 87). The average internal pH is also maintained at about 7.6 in light over a considerable range of external pH (12, 24, 34, 87, 88, 102), so that the magnitude of Ψ is variable. It is also influenced by medium composition.

MEMBRANE ORGANIZATION

The intracellular membrane systems of photosynthetic organisms are far more extensive than those of common heterotrophs, and the localization of transport systems needs to be considered in terms of the overall membrane organization that is described here briefly. Fuller accounts can be found (23, 36, 38, and 121). Excellent recent reviews (22, 112–114, 139, 141, 142, 144) give further background on the classification and physiology of the organisms.

Green Bacteria

The cytoplasmic membrane in these organisms contains the photosynthetic reaction centers (100), while the complex and exceptionally large light-harvesting antenna pigment arrays are internal to this membrane in the chlorosomes and intervening crystalline layer (32, 36, 140) (Figure 1A). Almost nothing is known about the entry or, for that matter, the exit of compounds from these organisms; it must be presumed, however, that transport systems are organized within the cytoplasmic membrane, which is not fully occupied by light energy–converting apparatus.

Purple Bacteria

Investigations on many strains have confirmed the membrane organization revealed by the classic studies of Holt & Marr (23, 51, 121), in which the continuities between the cytoplasmic and intracytoplasmic membrane systems were first demonstrated (Figure 1B). Because the extent of thylakoid development in Rhodospirillaceae can be regulated by exposure to different light intensities or oxygen concentrations, elegant studies of membrane biogenesis have been possible with these organisms (reviewed in 23, 63, 121). In several strains, differences in buoyant density and polypeptide composition between

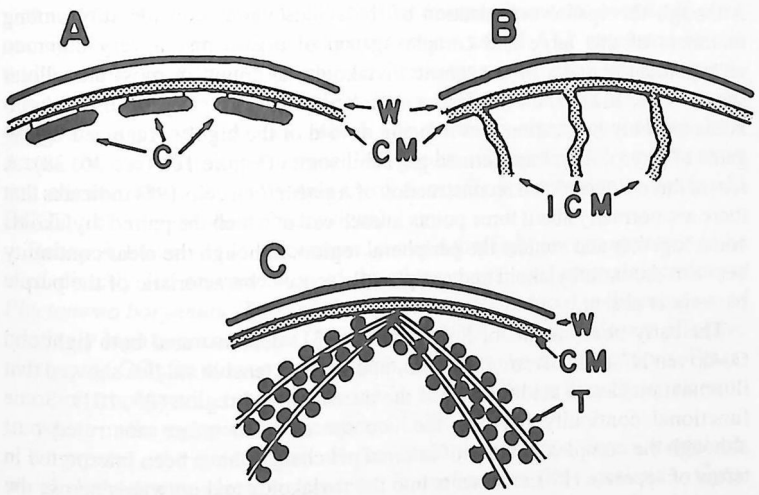


Figure 1 Generalized schematic drawing of membrane organization. *A*, green bacteria (Chlorobiaceae and *Chloroflexus*); *B*, purple bacteria (Chromatiaceae and Rhodospirillaceae); *C*, cyanobacteria (adapted from Ref. 98). *W*=wall (outer membrane and peptidoglycan layers); *CM*=cytoplasmic membrane; *C*=chlorosomes of green bacteria with crystalline layer connecting to cytoplasmic membrane; *ICM*=intracytoplasmic (thylakoid) membranes of purple bacteria; *T*=thylakoids of cyanobacteria with attached phycobilisomes.

the peripheral cytoplasmic and the intracytoplasmic membranes have been demonstrated (63), and a fraction that corresponds with nascent photosynthetic membrane has been identified (103).

The localization of transport components has not been clarified, but it seems reasonable to assume that they are located at least primarily in the cytoplasmic portions, as are penicillin-binding proteins (132). A consequence of this type of membrane organization is that the intrathylakoid space communicates directly with the periplasmic region. Protons are driven outwards during electron flow; consequently, inhibition of the ATPase concerned with photophosphorylation by dicyclohexylcarbodiimide (DCCD) results in increased acidification of the medium in illuminated cells (unpublished observations). The periplasmic volume and membrane surface is also very much greater than in an organism with uninvaginated membranes; for example, the sorbitol-excluding volume in *Chromatium vinosum,* corresponding to the true cytoplasmic space, is only 1.17 $\mu l \cdot mg\ protein^{-1}$ (111), as compared with 4–5 $\mu l \cdot mg^{-1}$ in common heterotrophs (145) or in unicellular cyanobacteria (34, 40, 54).

Cyanobacteria

Although the spatial organization of thylakoids varies considerably among members of this large and complex group of organisms, a very common arrangement consists of concentric thylakoids, as found in most unicellular species (38, 141). The cytoplasmic membrane differs clearly from the thylakoids not only in location, but in being devoid of the highly organized aggregates of phycobiliproteins termed phycobilisomes (Figure 1C) (see 36, 38). A recent three-dimensional reconstruction of *Agmenellum* cells (98) indicates that there are normally about three points in each cell at which the paired thylakoids come together and contact the peripheral region, although the clear continuity between the intrathylakoid and periplasmic spaces characteristic of the purple bacteria is absent.

The early observations of Scholes et al (131) demonstrated both light and O_2-driven H^+ efflux from *Anabaena,* and more extensive studies showed that illumination causes acidification of the intrathylakoid region (25, 101). Some functional continuity between the two spaces is therefore not ruled out, although the complex kinetics of internal pH changes have been interpreted in terms of separate H^+ movements into the thylakoids and outwards across the cytoplasmic membrane (5).

Recent reports on separation of different fractions of cyanobacterial membranes offer some promise of definitive identification of cytoplasmic membrane functions. Several procedures for isolation of outer regions of the wall have been published (94, 116, 122). These preparations, consisting primarily or entirely of outer membrane and peptidoglycan, contained little or no chlorophyll, although carotenoids were clearly present.

Membrane fractions relatively low in chlorophyll:protein ratios have been obtained from *Anacystis nidulans* (95, 100a) and from *Anabaena variabilis* (77). The latter preparations have an ATPase that does not react immunologically with antisera against spinach CF_1 and, even more interestingly, is inhibited by orthovanadate. Inhibition by this compound has been considered characteristic of plasmalemma ATPases of higher plants and other ATPases with phosphorylated intermediates (e.g. 106). The *Anabaena* fraction has also been shown to accumulate Ca^{2+} (78); if, as seems probable, inverted vesicles are formed in the course of preparation, this activity would be responsible for Ca^{2+} extrusion from whole cells.

A number of investigations, dealt with more fully later, have attempted to characterize cytoplasmic membrane functionally using whole cells, rather than fractionated preparations. The effect of temperature on rates of photosynthetic and respiratory electron flow yielded different Arrhenius plots, indicating that these functions have different microenvironments (108, 109, 129). Intact spheroplasts of *Anacystis nidulans* carried out an apparently cytochrome oxidase–mediated oxidation of exogenous cytochrome c (110), suggesting that the respiratory chains are located at least in part in the cytoplasmic membrane.

A number of investigations indicate that this membrane does not itself carry out light energy conversion, but rather makes use of ATP generated by the thylakoids to energize peripheral membrane functions. Thus low concentrations of DCCD strongly inhibit light-driven Na^+ export (104), and the uptake of glucose analogs by *Nostoc,* which was shown to depend on a pH gradient across the cytoplasmic membrane (93), is also inhibited. Both proton export and alkalinization of the cytoplasm of *Anacystis* are sensitive to 5–10 μM DCCD (34; unpublished observations).

Raboy & Padan (117), in contrast, showed that light energy appeared to be used directly for accumulation of the glucose analog α-methylglucoside in *Plectonema boryanum*. Total accumulation was not reduced in the presence of relatively high concentrations (50 μM) of DCCD, although this was effective in stopping CO_2 fixation or photophosphorylation in lysed preparations. Potassium ion transport in *Anabaena variabilis* also was driven directly by electron transport in the cytoplasmic membrane (120).

At present, the greater part of the available evidence favors models in which light-driven electron transport is confined to the thylakoids. Cytoplasmic membrane transport functions appear to be ATP-dependent in some genera, but not in all. The differences observed between organisms, perhaps to be expected in view of their phylogenic diversity (7), may possibly be related to varying levels of respiratory electron transport activity in the cytoplasmic membrane.

TRANSPORT OF NUTRIENTS

Carbon Source

INORGANIC CARBON

Anoxygenic bacteria The bulk of cell carbon for the green and red photosynthetic sulfur bacteria can, or even must, come from CO_2 (142). It might be expected that the environment of free-living anaerobes would be so rich in this compound that its supply and availability would pose no problems. This assumption may be questioned for the close-packed communities found in algal mats, where competition for CO_2 as well as for light and reduced sulfur compounds may restrict photosynthetic activity. In fact, values of 2–3 μM for the total inorganic carbon of interstitial pore water in sediment cores were recorded in a recent study (123).

As yet, however, movement of CO_2 or HCO_3^- into the cells of these organisms has not been studied. Anaerobic organisms using the reductive pentose phosphate cycle are, of course, shielded from the handicapped photosynthetic efficiency imposed by the dual substrate specificity of ribulose bisphosphate carboxylase/oxygenase, although glycolate excretion, the result of oxygenase activity, has been demonstrated both in *Chromatium* and in *Rhodospirillum rubrum* incubated in high concentrations of O_2 (80).

Oxygenic organisms Cyanobacteria inhabit environments where several factors contribute to give limited supplies of inorganic carbon. Many are benthic community dwellers, where oxygen levels can reach supersaturation and so affect the ability of ribulose bisphosphate carboxylase to fix CO_2. At the same time, the sharply raised pH often observed during daylight leads to a shift in the composition of inorganic pools towards HCO_3^- and CO_3^{2-}; these ions would not be expected to cross membranes without special mediation. Extensive discussion concerning bicarbonate transport in microalgae, both prokaryotic and eukaryotic, can be found in recent reviews (81, 119).

Clear evidence for bicarbonate ion transport requires unequivocal demonstration that the total rate of uptake exceeds the rate of formation of CO_2 from HCO_3^-, and therefore requires experimentation at low HCO_3^- concentrations (76). It must also be shown that the internal HCO_3^- concentration is greater than that outside the cell if active transport is inferred. In *Coccochloris peniocystis* (15, 90, 91), very short term experiments have demonstrated that inorganic carbon can be transported at a rate at least 10-fold greater than the spontaneous dehydration of HCO_3^-. A pool of up to 2.9 mM total inorganic carbon could be established internally, representing at least a 200-fold concentration difference across the cytoplasmic membrane. The difficulties of obtaining accurate values for specific activity of the internal pool, particularly in view of continuing respiration (130), makes the exact value hard to establish.

Light energy was required both for establishing and for maintaining the pool. The small pH gradient present under the experimental conditions could not account for the magnitude of accumulation, and a highly improbable membrane potential of 180 mV, positive internally, would be needed to achieve the measured concentration difference if HCO_3^- behaved as a permeant anion.

The filamentous cyanobacterium *Anabaena variabilis* has also been shown to accumulate inorganic carbon against an apparent concentration gradient (61). The high efficiency of carbon utilization by cells grown with low CO_2 could be accounted for by a transport system with an apparent K_m of about 150 μM. The affinity remained unchanged, but the maximum capacity of the system increased 10-fold during adaptation to low CO_2 in a process that required both energy and new protein synthesis (84, 85). Measurements of ΔpH and Ψ, negative inside, pointed to a rapid and sustained hyperpolarization when HCO_3^- was added to cells depleted of external inorganic carbon; transient K^+ influx was also recorded (62). Higher bicarbonate concentrations were employed in these studies than in those with *Coccochloris*, and a significant contribution from CO_2 to the total inorganic carbon transported was therefore possible.

ORGANIC CARBON

Amino acids The abilities of green and purple bacteria to incorporate small amounts of glutamate during growth (127) and, more dramatically, of purple bacteria to use amino acids as nitrogen source and partial carbon source (for example, 137) clearly implies that some amino acids can enter cells of photosynthetic organisms. Some of the more versatile cyanobacteria can also use amino acids (64, 96), and the incorporation of leucine by *Anacystis* measured by Smith et al (138) comes close to supplying the leucine needs for biosynthesis.

Amino acid auxotrophs of cyanobacteria have also been reported (e.g. 16, 20, 49, 135), implying some entry of amino acids. Studies dealing specifically with amino acid uptake or transport in photosynthetic bacteria have been important in developing current models of amino acid transport in systems that do not involve periplasmic binding proteins.

1. Purple bacteria: Leucine, valine, and alanine were incorporated by *Chromatium vinosum* during growth, but both the total capacities of the uptake systems and their affinities for substrates as measured in short-term experiments were low relative to the comparable transport systems in *E. coli* (154). It was suggested that cells were unable to accumulate a sufficiently high internal concentration to suppress biosynthesis. Alanine and its nonmetabolizable analog α-aminoisobutyrate (AIB) were taken up by a common light-driven,

protonophore-sensitive system (66, 67, 70, 71), and transport against a concentration gradient involved Na^+ symport (70, 111).

Because alanine uptake could also be demonstrated in spheroplasts, a periplasmic binding protein was not required (69). Interestingly, light-driven activity in spheroplasts was low unless the soluble cytochrome c_{551} was added. This confirms the periplasmic location of this component of the photosynthetic electron transport chain, and also provides a possible explanation for the low activity of membrane vesicles made from other organisms such as *Rhodopseudomonas sphaeroides* (see below).

Rapid uptake of alanine by the nonsulfur organism *Rps. sphaeroides* has been reported (47). Although special modifications of well-known methods were needed to make membrane vesicles that retained light-driven uptake ability, it was demonstrated that either light- or respiration-driven electron flow in such vesicles led to generation of a membrane potential, and that the accumulation of alanine was linked to this component of the proton-motive force.

Recent work with whole cells of *Rhodopseudomonas capsulata* (13, 57) indicates that membrane conductance is strongly influenced by the rate at which an electrochemical gradient is generated; relatively small changes in Ψ are observed until a critical rate of electron flow is reached. In *Rps. sphaeroides* vesicles (24) a static membrane potential would not serve to bring about alanine uptake, but electron flow must also occur; thus alanine is only taken up effectively under strongly energized conditions, and this "gating" effect is presumably one of the factors that led to the increased conductance seen in the whole cell experiments (57).

It would be of interest to know whether addition of exogenous cytochrome c_2, corresponding to the periplasmically located component that stimulated alanine uptake in the *Chromatium* spheroplasts (69), would affect the results of experiments with the *Rhodopseudomonas* systems, since its depletion might materially affect the integrity of the vesicles.

2. Cyanobacteria: Cyanobacterial active transport of an amino acid analogue was first shown in 1971 (28). The systems involved in amino acid transport have been more fully investigated recently (74, 75). In *Anacystis nidulans*, kinetic measurements suggest that a low- and a high-affinity system were involved in the uptake of alanine, glycine, or AIB, while a distinct system was responsible for the movement of the branched-chain amino acids leucine, isoleucine, and valine. The specificities of these energy-requiring uptake systems thus resembled those of many heterotrophs. No evidence for involvement of Na^+ in the high-affinity AIB uptake system was obtained, but the low-affinity system was indeed stimulated by Na^+.

The experiments suggest that the uptake of AIB, at least, is electrogenic,

involving proton symport, but the complexity of the ionic and pH effects makes further clarification desirable. Although a number of assumptions have to be made in calculating rates, it does appear that a major part, perhaps half, of the leucine needed for protein synthesis during growth of the cells could be met by the maximum activity of the leucine uptake systems. The filamentous organism *Anabaena variabilis* also is able to take up glutamate and glutamine from low external concentrations (11); high-affinity and low-affinity systems appear to be present, with K_m of the order of 25–100 μM and 3–5 mM respectively.

Interestingly, the high-affinity systems appeared to share a common component, and there was also an indication that the glutamine analog methionine sulfoximine entered the cells by the same transporter. The V_{max} of these high-affinity systems does not appear to be great enough to provide more than about one tenth of the amino acid nitrogen required for growth, which may explain the failure of single amino acids to support growth in the absence of inorganic nitrogen sources.

Carboxylic acids

1. Monocarboxylates: Of all compounds tested, acetate is the organic molecule most widely used by phototrophs. Even those whose metabolism is highly geared to using CO_2 as their main carbon source, such as the green bacteria and cyanobacteria, will incorporate substantial quantities of acetate during growth; the actual proportion of cell carbon that can be provided is limited primarily by the metabolic pathways employed by the organisms. It has been observed repeatedly that acetate can be removed almost quantitatively from the growth medium (eg. 54, 127). Saturation kinetics were seen in short-term uptake experiments with the cyanobacteria, but the kinetic constants were remarkably pH-dependent (54). Mutants unable to incorporate acetate accumulated acetate reversibly, to an extent that depended on the pH difference between the cells and the medium in which they were suspended, and attempts to show the involvement of a carrier in acetate movements were unsuccessful (34).

It appears reasonable to assume that acetic acid can enter cyanobacteria freely, while acetate ion is relatively impermeant. This simple uptake mechanism allows the intracellular concentration to become 8–10 times higher than the external, provided that cells are in neutral or slightly acid environments, but also hinders acetate utilization at alkaline pH. There is at present no reason to suppose that other fatty acids require more elaborate means for entering cells of any kind. The ready release of these acids as fermentation end products, and their damaging effects in, for example, *Rps. capsulata* (97), may be explained by the ease of the movement of the protonated forms.

2. Pyruvate: Pyruvate has been found to contribute a fraction of cell carbon in a number of chemo- and phototrophs, including green sulfur and cyanobacteria

(127, 136, 138, 139). It is widely used as a sole carbon source by Chromatiaceae and Rhodospirillaceae, including strictly fermentative growth of *R. rubrum* (39, 149). The light-dependent uptake of pyruvate by actively metabolizing cells of *Rps. sphaeroides* was kinetically complex, allowing clear definition only of a high-affinity system (K_m 13 μM; V_{max} 5.6 nmol·min^{-1}·mg protein $^{-1}$) that could contribute no more than about 10% of the total carbon needs of growing cells (33). This suggests that a low-affinity system, which did not saturate, was of considerable importance in supplying cellular needs.

3. Dicarboxylates: Malate uptake by *Chromatium vinosum,* like that of alanine, was found to be insensitive to DCCD in light, but eliminated by protonophores, indicating that the electrochemical gradient of protons appeared to be driving its uptake (66). Quoted rates for malate uptake appear too low to satisfy growth needs; the system does not appear to have been characterized further. An inducible uptake system apparently able to bring carbon into the cells fast enough to account for the observed growth rate was demonstrated in *Rps. sphaeroides* (33); a common system for malate, succinate, and fumarate was indicated by the mutual interference of one of these compounds with the uptake of another.

Because metabolite diversification of entered dicarboxylate was rapid, clear evidence for establishment of a concentration gradient between the cells and their environment was not obtained. In contrast to purple bacteria, cyanobacteria incorporated at most traces of succinate during growth, and entry of dicarboxylates was not detected by the silicone barrier centrifugation method (34).

4. Glycolate: Movements of glycolate are of special interest, in view of its production by organisms employing the reductive pentose phosphate pathway of CO_2 fixation in high oxygen tensions (80, 146). It seems to be generally assumed that entry, and exit if glycolate is produced intracellularly, is by nonspecific leakage across the cell membrane, and no evidence for saturation of incorporation or of entry rates in unicellular cyanobacteria was obtained (34, 89).

5. Neutral compounds: Small neutral molecules such as ethylene glycol are widely used as permeant indicators of intracellular volume (126). Although intracellular volume obtained with this probe is about 10% less than using ^3H_2O, penetration of the molecule appears complete within 30 sec with *Chromatium, Rps. sphaeroides,* and several unicellular cyanobacteria (unpublished observations). Glycerol, which can support photoheterotrophic growth of *Rps. sphaeroides* and *Rps. capsulata* (82, 115), likewise seems to enter rapidly enough to supply growth needs by a process of simple diffusion, and no evidence for a facilitated system such as exists in *E. coli* (124) was found.

Sugars As noted earlier, sugars generally are not especially favored growth substrates of even the preferentially photoheterotrophic bacteria, although a number of Rhodospirillaceae may in fact use fructose as sole carbon source. Indeed, glycolytic breakdown of fructose can support anaerobic growth of *Rps. capsulata* in darkness, provided an alternative electron acceptor such as DMSO is present (83).

1. Purple bacteria: Glucose, or its nonmetabolizable analog α-methylglucoside, was accumulated by a common energy-dependent, saturable process by *Chr. vinosum* (68). The entered sugars were not phosphorylated, and transport appeared to be driven directly by the electrochemical gradient produced during illumination. The maximum uptake rate, at least of the analog, could supply only about 5% of the carbon needs for growth, however.

In contrast to *Chromatium, Rps. sphaeroides* and *R. rubrum* possess a phosphoenolpyruvate-dependent (PTS) uptake system for fructose (128), the sugar most readily used by these organisms. The uptake rate of 4.5 μmol·min^{-1}· mg dry wt^{-1} was more than adequate to provide for cell carbon needs for biosynthesis and also for estimated requirements for energy generation during aerobic growth in darkness.

2. Cyanobacteria: Although many reports of low levels of sugar incorporation by cyanobacteria have appeared (28, 107, 138), in most instances high concentrations of sugars seemed to be needed for significant incorporation. More detailed studies showed that *Aphanocapsa* and *Nostoc* carried out energy-dependent 100-fold accumulation of 3–0-methyl glucose from external concentrations of 20 μM (6). This nonmetabolizable analog, 6-deoxyglucose, and glucose itself were taken up by a common system, as judged from competition and chase experiments.

Although the maximum capacity of this uptake system could indeed provide for the cells' carbon needs both photo- and chemoheterotrophically, it had a relatively high K_m of about 0.4 mM. The entry of the sugar was shown to be dependent on a pH gradient between the cells and their surroundings, applied artificially in energy-poisoned cells (93). These experiments also indicated clearly that the production of this gradient required photosynthetic or oxidative phosphorylation, and that it could not be generated directly by light-driven or respiratory electron flow.

Another filamentous cyanobacterium, *Plectonema boryanum,* which is able to grow chemoheterotrophically with several sugars, was shown to have an inducible ability to accumulate glucose or α-methylglucoside when the cells were energized either by illumination or respiration (117). Accumulated α-methylglucoside was not chemically modified and was rapidly released on adding glucose, unlabelled α-methylglucoside, or a protonophore. Either com-

ponent of Δp was able to support uptake, which therefore appeared to be an electrogenic process involving proton symport.

In this organism, DCCD did not affect the total accumulation of the sugar analog; a proton-motive force across the cytoplasmic membrane was therefore generated without the intermediate form of ATP as in *Anabanea*, and in contrast with *Nostoc*. Glucose transported by this system working at its maximum rate could support the dark heterotrophic growth rate of *Plectonema* and gave some support to the suggestion that this low rate is due to slow entry of the substrate. More than one transport system must be present in this organism, since other sugars that will support growth did not compete with α-methylglucoside for entry. Fructose, for example, is used for growth by *Nostoc* (9) and stimulated growth and nitrogen fixation in *Anabaena variabilis* (45). The apparent affinity of the *A. variabilis* uptake system increased with time of exposure to the sugar: final $K_m \sim 50$ μM, a value substantially lower than those for glucose entry in *Nostoc* and *Plectomema*.

Sugar transport systems thus seem to be more widespread among phototrophs than is the ability to grow with sugars as the sole carbon source. A phosphotransferase system has so far been found only among a small number of Rhodospirillaceae, and although its regulation has not been examined, its activity appears well able to supply growth requirements. Among cyanobacteria, the electrogenic uptake systems described could supply what is needed for the observed maximum growth rates, but the affinities of the systems appear to be relatively low and raise doubts about the occurence of chemoheterotrophic growth (using sugars) in any natural situation. Both the capacity and affinity of sugar uptake raise the same questions about *Chromatium*.

Nitrogen Source

The greater number of anoxygenic phototrophs are potentially able to fix gaseous nitrogen (112, 114, 142, 144), and this ability is found also among heterocystous and a small number of nonheterocystous cyanobacteria kept under anaerobic or microaerophilic conditions (125). It is difficult to imagine any supply problem for the green and purple bacteria using this mode of cell nitrogen acquisition, since their anaerobic environments are likely to be saturated or even supersaturated with N_2. The possibility that the properties of the heterocyst wall may lead to some restriction in N_2 entry has been raised by Raven (119), but does not appear to have been tested experimentally.

AMMONIA It is, of course, clearly established that nitrogenase activity leads to the production of ammonia, which is subsequently incorporated into organic molecules. Since N_2 reduction is a costly process in terms of energy, a high efficiency of incorporation is to be expected, at least in free-living forms; processes leading to release of fixed nitrogen, presumably as ammonia, in return for supplies of other nutrients may have been secondarily selected for in

symbiotic associations. These considerations, and the fact that free ammonium is almost invariably used in preference to any other nitrogen source, have prompted a number of investigations concerning the movement of ammonia into and out of cells of both free-living and symbiotic phototrophs, mostly employing the convenient radioactive analog methylammonium.

Purple bacteria Light-dependent, uncoupler-sensitive uptake of $^{14}CH_3NH_3^+$ has been observed in a number of Rhodospirillaceae (1, 2, 56). The uptake of this ammonium analog was depressed if cultures were grown in high concentrations (15 mM) of NH_4^+, when the growth needs of the cells could presumably be met by diffusive entry. The pH profile of $CH_3NH_3^+$ uptake, which exceeded what could be expected for a pH-dependent distribution such as has been characterized for polyamines (41), suggests that the protonated ion, rather than the free amine, was transported. However, considerable metabolic conversion of the entered analog can be inferred from the incomplete removal of radioactivity from the cells in chase experiments (see also 37, 156). Ammonium was considered to be the natural substrate, since it inhibited $CH_3NH_3^+$ uptake competitively (K_m for $CH_3NH_3^+$, ~100 μM; K_i for NH_4^+, ~10 μM).

Cyanobacteria Cyanobacteria are known to take up ammonia in preference to other usable N sources (99). Internal pools of NH_4^+, not bound to macromolecules, amounting to 2–3 mM were found in *Anacystis* during exponential growth or after prolonged nitrogen starvation (Boussiba et al, submitted for publication). The cells took up NH_4^+ in light against apparent concentration gradients of 10^3, based on total internal volume. The apparent K_m of this uptake was less than 0.5 μM, and the V_{max} of 25–35 nmol·min^{-1}·mg protein^{-1} was more than adequate to supply growth needs of N. This ability would account for the lack of detectable ammonia excretion during growth on nitrate (40) by recapture of NH_4^+ if any outward diffusion occurred.

However, NH_4^+ release has been observed on addition of the glutamine synthetase inhibitor, methionine sulfoximine, to suspensions of cyanobacteria incubated with (118, 143) or without nitrate (Boussiba et al, submitted for publication), with at most a two-fold increase in intracellular ammonia concentration. Whether this release involves overriding or reversing a normal uptake mechanism, or whether a different process is involved, is not yet clear. Internal pools and uptake rates were found to be maintained over a pH range of about 3 units, which would involve an almost 1000-fold change in the ratio of NH_3/NH_4^+ externally, with little effect on internal pH in light (26, 34). The diffusion of NH_3 across the cytoplasmic membrane of *Anacystis* and related organisms thus appears to be extraordinarily low, but has not been measured directly. A similar conclusion was drawn from studies with nonphotosynthetic bacteria (65).

NITRATE Few of the purple bacteria use nitrate either as nitrogen source or as electron acceptor (142), and in those that do the entry process has not been studied. Cyanobacteria, on the other hand, are all able to use NO_3^-, and removal of this ion from culture medium can be close to quantitative (e.g. 40, 159). The nature of the transport system involved in its entry into the cells has not been clarified.

The entry of amino acids, as important in terms of nitrogen as of carbon source, has been considered in that section.

Phosphate

Unlike the possible sources of cell carbon, nitrogen and sulfur, phosphorus needs are supplied solely by the uptake of inorganic phosphate (46, 119). A number of microorganisms, including some phototrophs, are able to use organic phosphates for growth, but the available evidence suggest these must first be hydrolyzed by periplasmically located phosphatases, as has been found in unicellular cyanobacteria (53).

Investigations of phosphate transport involving anoxygenic phototrophs are limited, although phosphate may well be a nutrient in short supply in the environment that they inhabit. In *Rps. sphaeroides* vesicles, an uncoupler-sensitive, carrier-mediated uptake of inorganic phosphate allowed the establishment of a 29-fold difference in Pi_{in}/Pi_{out} (48). The component of Δp needed for the accumulation varied with external pH; Ψ was clearly involved at pH 8, but ΔpH also contributed at pH 6. These findings may reflect the variation in the components of Δp when external pH is altered (102). Whether transport characteristics were modulated by growth conditions in this organism does not appear to have been investigated.

In contrast, a very large number of studies have involved cyanobacteria, particularly the unicellular ones, with findings that are not entirely consistent. An energy- and divalent cation–dependent uptake has been reported in *Anacystis nidulans* (8, 25, 27). The cation dependence was not seen, although not rigorously excluded, in washed cells of a different strain of *Anacystis* taken from phosphate- or nitrate-limited continuous culture (40) in which an apparent concentration difference of 10^3 was found between the inside of the cells and their surroundings.

As pointed out by Simonis and colleagues (27), it is essential to establish whether intracellular phosphate is free or bound before postulating that active transport is involved. From kinetic and other considerations, these authors concluded that phosphate must not only enter together with divalent cations, but that it was bound to such cations internally to the extent that no significant free ionic concentration difference exists across the cytoplasmic membrane. A complex model, involving facilitated diffusion to a degree dependent on internal Mg^{2+}, whose concentration was itself modulated by movements between the cytoplasmic and intrathylakoid spaces, was suggested. A more conventional explanation appears adequate to account for phosphate uptake in the other strain, in which over 90% of entered radioactivity was recovered as inorganic phosphate in the first minute or less, and phosphate-limited growth induced a 50-fold increase in V_{max} without change in the apparent K_m of 0.2 μM (40).

Sulfur Source

Sulfur compounds occupy a special place in the metabolism of photosynthetic bacteria, being in some cases required as major electron donors for CO_2 reduction, but in all cases needed also in the biosynthesis of sulfur-containing amino acids and cofactors.

HYDROGEN SULFIDE The most generally used electron donor among the autotrophic phototrophs is H_2S, although this compound is also among the more toxic natural products for all forms of life. In his early and classical studies, van Niel (153) concluded that the degree of toxicity of H_2S to the green and purple sulfur bacteria depended on pH, and therefore on the concentration of the protonated form. The toxicity of sulfide to cyanobacteria (52) was also correlated with the concentration of H_2S, which entered the cells very rapidly. It is characteristic of almost all sulfide-utilizing phototrophs that sulfide is oxidized to S° more rapidly than S° is further oxidized to SO_4^{2-}.

In Chlorobiaceae, but among purple organisms only in *Ectothiorhodospira*, the S° is converted to insoluble aggregates extracellularly. In all other remaining Chromatiaceae, S° is retained within the cell boundary in granules that are surrounded by a characteristic non–unit membrane layer (121, 147). It is at least possible that sulfide, protonated or unprotonated, is in all cases oxidized on the exterior face of the peripheral cytoplasmic or intracytoplasmic membrane, and that S° aggregates form within the periplasmic region in most *Chromatiaceae*, sequestering potential electron donors from competitive organisms. The resistence of an organism to high sulfide would thus be a function of the success of the cytoplasmic membrane as an oxidative barrier for protecting the cell from its toxic effects.

Cytochromes that are solubilized on cell disruption are involved in sulfide oxidation (147), and it would be interesting to know if any are located in the periplasm, as has been found in sulfate-reducing bacteria (105). When sulfide is used as electron donor for anoxygenic photosynthesis by the cyanobacterium *Oscillatoria limnetica* (14), elemental sulfur is deposited outside the cells, and is not oxidized further. There is a very wide variation in the tolerance of different cyanobacteria for sulfide, which might be correlated with the rate at which this compound can be oxidized.

ELEMENTAL SULFUR Exogenous elemental sulfur can be oxidized to sulfate by green and purple bacteria, and the initial step in oxidation of S° thus most probably occurs on the outer face of the cytoplasmic membrane. The pathways involved in oxidation have been extensively investigated by Trüper and colleagues (147, 148). In Chlorobiaceae and the greater number of Chromatiaceae, adenylyl sulfate (APS) is formed, and this reaction presumably occurs in the cytoplasmic compartment where contact between the soluble APS reductase and its substrates can occur. However, neither the chemical form in which partially oxidized sulfur compounds cross the cytoplasmic barrier, nor the mechanism involved, has been clarified. The same is true for the exit of the end product of oxidation, SO_4^{2-}.

SULFATE The initial steps of assimilatory sulfate reduction clearly involve APS or 3'-phosphoadenylyl sulfate (PAPS) formation by soluble cytoplasmic enzymes (147, 148), and so must be preceded by entry of SO_4^{2-} into the cells. In anoxygenic organisms, the sulfate entry process has not been studied in any detail. *Rhodopseudomonas globiformis,* previously thought to be unable to assimilate SO_4^{2-} (142), could in fact do so if the medium concentration was kept below 0.8 mM (55). Repression of a SO_4^{2-} transport system, as well as of the enzymes of the reductive steps in assimilation, was suggested to explain these findings.

Two groups have investigated SO_4^{2-} uptake by the cyanobacterium *Anacystis nidulans*. Sulfate uptake was shown to require light and to be inhibited in the presence of protonophores (60), and the rate of uptake was increased 40-fold by 4–5 hours' incubation of suspensions in light but without SO_4^{2-}. A high affinity (K_m 0.75 μM) for uptake in SO_4^{2-}-starved cells with unusual kinetics was observed (150); both SO_3^{2-} and $S_2O_3^{2-}$ inhibited uptake competitively, suggesting a common uptake system. The internal concentration calculated from acid soluble counts exceeded the external by at least 20-fold, but the chemical state of the radioactive pool was not determined. These studies therefore do not indicate unequivocally whether SO_4^{2-} entry into *Anacystis* is by facilitated or active transport.

Inorganic Cations

The inorganic cation requirements of photosynthetic organisms, as of all bacteria, can be grouped according to function; some, for example iron, become incorporated into organic molecules, and their uptake is essentially irreversible. Others, such as Na^+ and K^+, are presumably unbound in the cytoplasmic compartment, are cycled continuously across the cytoplasmic membrane during metabolic activity and growth, and are involved in maintaining osmotic balance and in regulating internal pH (19, 102). Of the divalent cations, magnesium appears to be freely exchangeable in *Rps. capsulata* (59), but may cycle less freely in cyanobacteria, being largely bound to inorganic phosphate (27) and macromolecules.

SODIUM Movements of Na^+ have been studied in whole cells and spheroplasts of *Chromatium* (19, 69, 70) partly in connection with the uptake of sugars and amino acids as noted in those sections. Sodium was driven out of cells by an energy-dependent, protonophore-sensitive mechanism; as a clear case for Na^+/H^+ antiport could not be made, the coupling may still be indirect. *Rhodopseudomonas sphaeroides* chromatophores took up Na^+ through an electroneutral exchange for protons (48). In *A. nidulans,* extrusion of Na^+ was DCCD-sensitive, but driven through a proton-translocating ATPase and subsequent Na^+/H^+ antiport (104).

POTASSIUM The internal concentration of K^+ is normally much higher than the external, in photosynthetic as in other organisms. In *Chromatium,* the internal K^+ varied in relation to the external ionic concentration, suggesting that it is involved in regulating osmotic pressure (19, 71). An ATP-driven K^+ uptake system was demonstrated, and in addition a system for K^+/H^+ exchange (17). Similar systems were found in whole cells of *Rps. capsulata* (58).

A detailed study of K^+ movements in *Anabaena variabilis* (120) led to the conclusion already noted in the section on membrane organization, that electron flow in the cytoplasmic membrane was responsible for direct production of Δp. The K^+ accumulation potential was always about 20 mV more negative than the directly measured membrane potential unless DCCD was present, suggesting that a primary ATP-driven K^+ pump was involved in intact cells. A similar conclusion was reached about K^+ accumulation in *A. nidulans* (104). The complexities of monovalent cation movements reflect their many functions in cellular homeostasis as well as symport with other nutrients.

CALCIUM Divalent cation gradients are also maintained by photosynthetic organisms. Energy-dependent Ca^{2+} extrusion has been demonstrated in whole cells and spheroplasts of *Chromatium* and *R. rubrum* (17–19), the mechanism being most probably an electrogenic Ca^{2+}/H^+ antiport. The characteristics of a presumably inverted membrane vesicle preparation from *Anabaena variabilis* that carries out an ATP-dependent Ca^{2+} accumulation (78) have been mentioned in the section on membrane organization.

MAGNESIUM Light- or respiration-dependent magnesium uptake has been demonstrated in *Rps. capsulata* (59). Accumulated Mg^{2+} exchanged slowly with extracellular ion during growth, but was liberated within 30 min on incubation with DCCD in the absence of external Mg^{2+}, suggesting that retention required continued presence of ATP. A separate system was impli-

cated in the uptake of Mn^{2+}. The apparent K_m of both (55 μM for Mg^{2+}; 0.5 μM for Mn^{2+}) appeared to be low enough for the uptake systems to be significant in natural environments. The Mg^{2+} requirement of *Anacystis* has been studied in continuous culture (151, 152). The internal concentration calculated from the cell-yield curve varied from 27–78 mM when the medium input was 2.5–10 μM, implying very effective uptake. As noted earlier in discussion of phosphate transport, the bulk of these ions is probably sequestered internally, so that the free-ion concentration difference may be exaggerated.

IRON Iron is an important functional constituent of the electron transport chains of photosynthetic and of nonphotosynthetic organisms. In anaerobic, sulfide-containing environments, the bulk of the iron will exist as Fe^{2+}, and be precipitated as the extremely insoluble FeS. How iron is brought into these cells against such competition does not appear to have been examined. Cyanobacteria, inhabiting generally highly aerobic environments, need to acquire iron from equally insoluble ferric compounds.

Both filamentous (3) and unicellular (134) cyanobacteria have been shown to produce citrate-hydroxamate siderophores termed schizokinens. A saturable, energy-dependent uptake of ferric schizokinen with an apparent K_m of 40pM was found that was blocked both by protonophores and by DCCD and thus appeared to be driven by ATP, although the mediation of a Δp was not excluded (72). The reductive release of Fe^{2+} from the Fe^{3+}-schizokinen has not yet been studied.

Inorganic Anions

By comparison with cation movements, little is known about anion uptake or expulsion. A tentative suggestion of active uptake of Cl^- by *Anacystis* (21) has been confirmed in a more recent study (158), in which energy-dependent accumulation of $^{36}Cl^-$, with apparent K_m of 20 μM, led to 160-fold higher concentration inside the cells than out. Uptake was maximal at low external pH, which emphasized the ΔpH component of Δp, rather than Ψ, and was both protonophore- and DCCD-sensitive. The findings favored a model involving a primary ATP-utilizing pump.

CONCLUDING REMARKS

The transport systems of photosynthetic bacteria, whether oxygenic or anoxygenic, appear to be mechanistically very similar to those of the rather limited number of heterotrophic organisms that have been used for in-depth studies—primarily *E. coli, B. subtilis,* a few pseudomonads, and *Streptococcus faecalis.* The same classes of systems are found, though not always generally distrib-

Simple diffusion appears adequate to account for entry of glycerol and simple fatty acids.

Many investigations that suggest involvement of a carrier in uptake of a nutrient do not at present allow clear distinction between a facilitated and active transport system, because chemical modification of entered substrate or even analog is rapid. The possibility of internal compartmentation, particularly in cyanobacteria, has received little attention. Proton motive force generated either directly by electron flow or indirectly by ATP hydrolysis is, however, clearly involved in the active uptake of a number of nutrients. The components of Δp may be involved directly in sugar import or in Na^+ extrusion; secondary coupling through Na^+ gradients and possible other intermediates may also occur. Primary movements of K^+ have been observed in several types of organism. Systems with relatively broad specificity have been implicated in amino acid transport. Specificity appears higher for sugar uptake, whether H^+ gradient–linked or involving group translocation, but the phosphotransferase system does not appear to be widely distributed. Inducibility is evident in some systems, for instance those involved in phosphate and sulfate uptake, but is less apparent than among the organic compound transport systems of heterotrophs.

The similarities in transport mechanism are not entirely surprising in a diverse collection of organisms that may share common ancestors (31) as well as be open to gene transfer in natural environments (86). Similarities are also to a considerable degree built in to the experimental approaches taken in a great number of the investigations reviewed in this article, which are modeled heavily on findings with heterotrophs. Some transport problems particularly important to anoxygenic organisms, especially in relation to the transport of sulfur compounds, have yet to be explored.

If the mechanisms involved in nutrient transport systems seem to be fairly similar in all organisms, there are some discernible differences in nutrient acquisition strategy of phototrophs and the more familiar fast-growing heterotrophs. It is true that the high-capacity, usually inducible, transport systems specialized for massive accumulation of individual organic molecules have a few parallels among the Rhodospirillaceae, which can indeed adopt an aerobic heterotrophic life-style that would bring them into direct competition with obligate organotrophs. But among other types such as Chlorobia, many Chromatiaceae, and cyanobacteria, uptake of individual organic molecules may be modest and inadequate for total carbon needs, though encompassing a broad range of compounds. Their utilization rarely requires preadaptation, and uptake appears to depend on relatively unspecific transport systems. Some of the systems for the uptake of carbon sources other than sugars appear to have high affinities for their substrates, so that a measure of mixotrophy may be the standard rather than exceptional in natural environments.

Since light energy is essentially a periodic energy source, and cells must

survive hours of darkness, the storage abilities of these organisms are exceptionally well developed. Their transport abilities, as well as their biosynthetic strategies, appear geared towards opportunistic uptake and polymerization of nutrients. These function as intracellular sources for later synthesis of the complex macromolecules that constitute working cell machinery and also serve as energy source during the night. Accumulation of other growth requirements needed in less massive amounts, such as sulfur and phosphorus sources, clearly involves the ability to compete with other bacteria on equal terms, because the needs for all physiological types are quantitatively similar. Again, storage abilities are well marked, notably in the form of polyphosphates, aiding in episodic growth.

An important function of at least some of the transport abilities discussed in earlier sections may be in retention of molecules generated intracellularly, which must have a finite tendency to be lost by outward diffusion. Photosynthetic organisms, with their extensive abilities to use the simplest sources of carbon and nitrogen for complex biosyntheses, should prove particularly valuable in evaluating this possibility genetically. The recent and dramatic progress in developing genetic systems in anoxygenic bacteria (86, 137, 157) and in cyanobacteria (10, 16, 32, 50, 133) offers promise for rapid progress in examining the physiological function as well as the molecular make-up of transport systems in these organisms.

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Literature Cited

1. Alef, K., Kleiner, D. 1982. *Arch. Microbiol.* 132:79–81
2. Alef, K., Kleiner, D. 1982. *Arch. Microbiol.* 133:239–41
3. Armstrong, J. E., Van Baalen, C. 1979. *J. Gen. Microbiol.* 111:253–62
4. Baccarini-Melandri, A., Casadio, R., Melandri, B. A. 1981. *Curr. Top. Bioenerg.* 12:197–258
5. Barsky, E. L., Gusev, M. V., Nikitina, K. A., Samuilov, V. V. 1981. *Arch. Microbiol.* 129:105–8
6. Beauclerk, A. A. D., Smith, A. J. 1978. *Eur. J. Biochem.* 82:187–97
7. Bonen, L., Doolittle, W. F. 1979. *Can. J. Biochem.* 57:879–88
8. Bornefeld, T., Simonis, W. 1974. *Ber. Deutsch. Bot. Gesellsch.* 87:493–500
9. Bottomley, P. J., Van Baalen, C. 1978. *J. Bacteriol.* 135:888–94
10. Buzby, J. S., Porter, R. D. Stevens, S. E. Jr. 1983. *J. Bacteriol.* 154:1446–50
11. Chapman, J. S., Meeks, J. C. 1983. *J. Bacteriol.* 156:122–29
12. Clark, A. J., Jackson, J. B. 1981. *Biochem. J.* 200:389–98
13. Clark, A. J., Cotton, N. P. J., Jackson, J. B. 1983. *Eur. J. Biochem.* 130:575–80
14. Cohen, Y., Padan, E., Shilo, M. 1975. *J. Bacteriol.* 123:855–61
15. Coleman, J. R., Colman, B. 1981. *Plant Physiol.* 67:917–21
16. Currier, T. C., Haury, J. F., Wolk, C. P. 1977. *J. Bacteriol.* 129:1556–62
17. Davidson, V. L., Knaff, D. B. 1981. *Photobiochem. Photobiophys.* 3:167–74
18. Davidson, V. L., Knaff, D. B. 1982. *Photochem. Photobiol.* 36:551–58
19. Davidson, V. L., Knaff, D. B. 1982. *Arch. Biochem. Biophys.* 213:358–62

20. Delaney, S. F., Herdman, M., Carr, N. G. 1976. In *The Genetics of Algae*, ed. R. A. Lewin, pp. 7–28. Oxford: Blackwell
21. Dewar, M. A., Barber, J. 1974. *Planta* 117:163–74
22. Doolittle, W. F. 1979. *Adv. Microb. Physiol.* 20:1–102
23. Drews, G., Oelze, J. 1981. *Adv. Microb. Physiol.* 22:1–92
24. Elferink, M. G. L., Friedberg, I., Hellingwerf, K. J., Konings, W. N. 1983. *Eur. J. Biochem.* 129:583–87
25. Falkner, G., Werdan, K., Horner, F., Heldt, H. W. 1976. *Ber. Deutsch. Bot. Gesellsch.* 89:285–88
26. Falkner, G., Horner, F., Werdan, K., Heldt, H. W. 1976. *Plant Physiol.* 58:717–18
27. Falkner, G., Horner, F., Simonis, W. 1980. *Planta* 149:138–43
28. Faust, G. R., Orcutt, A. R., Shearin, S. J. 1971. *Planta* 100:360–64
29. Ferguson, S. J., Jones, O. T. G., Kell, D. B., Sorgato, M. C. 1979. *Biochem. J.* 180:75–86
30. Ferguson, S. J., Sorgato, M. C. 1982. *Ann. Rev. Plant Physiol.* 51:181–217
31. Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., et al. 1980. *Science* 209:457–63
32. Gendel, S., Straus, N., Pulleyblank, D., Williams, J. 1983. *J. Bacteriol.* 156: 148–54
33. Gibson, J. 1975. *J. Bacteriol.* 123:471–80
34. Gibson, J. 1981. *Arch. Microbiol.* 130: 175–79
35. Deleted in proof
36. Glazer, A. N. 1983. *Ann. Rev. Biochem.* 52:125–57
37. Gober, J. W., Kashket, E. 1983. *J. Bacteriol.* 155:1196–1201
38. Golecki, I. R., Drews, G. 1982. *The Biology of Cyanobacteria*, ed. N. G. Carr, B. A. Whitton, pp. 125–41. Berkeley: U.C. Press
39. Gorrell, T. E., Uffen, R. L. 1977. *J. Bacteriol.* 131:533–43
40. Grillo, J. F., Gibson, J. 1979. *J. Bacteriol.* 140:508–17
41. Guarino, L. A., Cohen, S. S. 1979. *Proc. Natl. Acad. Sci. USA* 76:3184–88
42. Harold, F. M. 1972. *Bacteriol. Rev.* 36:172–230
43. Harold, F. M. 1977. *Curr. Top. Bioenerg.* 6:84–149
44. Harold, F. M. 1982. *Curr. Top. Membr. Transp.* 16:485–516
45. Haury, J. F. Spiller, H. 1981. *J. Bacteriol.* 147:227–35
46. Healey, F. P. 1982. See Ref. 38, pp. 105–24
47. Hellingwerf, K. J., Michels, P. A. M., Dorpema, J. W., Konings, W. N. 1975. *Eur. J. Biochem.* 55:397–406
48. Hellingwerf, K. J., Friedberg, I., Lolkema, J. S., Michels, P. A. M. Konings, W. N. 1982. *J. Bacteriol.* 150:1183–91
49. Herdman, M., Delaney, S. F., Carr, N. G. 1973. *J. Gen. Microbiol.* 79:233–37
50. Herdman, M. 1982. See Ref. 38, pp. 263–306
51. Holt, S. C., Marr, A. G. 1965. *J. Bacteriol.* 89:1402–12
52. Howsley, R., Pearson, H. W. 1979. *FEMS Microbiol. Lett.* 6:287–92
53. Ihlenfeldt, M. J. A., Gibson, J. 1975. *Arch. Microbiol.* 102:23–28
54. Ihlenfeldt, M. J. A., Gibson, J. 1977. *Arch. Microbiol.* 113:231–41
55. Imhoff, J., Then, J., Hashwa, F., Trüper, H. G. 1981. *Arch. Microbiol.* 130:234–37
56. Ivanovsky, R. N., Vakulenko, V. P., Kondratieva, E. N. 1982. *4th. Int. Symp. Photosynth. Prokaryotes, Bombannes, France*
57. Jackson, J. B. 1982. *FEBS Lett.* 139:139–42
58. Jasper, P. 1978. *J. Bacteriol.* 133:1314–22
59. Jasper, P., Silver, S. 1978. *J. Bacteriol.* 133:1323–28
60. Jeanjean, R., Broda, E. 1977. *Arch. Microbiol.* 114:19–23
61. Kaplan, A., Badger, M. R., Berry, J. A. 1980. *Planta* 149:219–26
62. Kaplan, A., Zenvirth, D., Reinhold, L., Berry, J. A. 1982. *Plant Physiol.* 69: 978–82
63. Kaplan, S. 1981. *Photochem. Photobiol.* 34:769–74
64. Kapp, R., Stevens, S. E. Jr., Fox, J. L. 1975. *Arch. Microbiol.* 104:135–38
65. Kleiner, D. 1981. *Biochim. Biophys. Acta* 639:41–52
66. Knaff, D. B. 1978. *Arch. Biochem. Biophys.* 189:225–30
67. Knaff, D. B., Whetstone, R., Carr, J. W. 1979. *FEBS Lett.* 99:283–86
68. Knaff, D. B., Whetstone, R. 1980. *Arch. Biochem. Biophys.* 203:697–701
69. Knaff, D. B., Whetstone, R., Carg, J. W. 1980. *Biochim. Biophys. Acta* 590: 50–58
70. Knaff, D. B., Davidson, V. L., Pettitt, C. A. 1981. *Arch. Biochem. Biophys.* 211:234–39
71. Knaff, D. B., Davidson, V. L. 1982. *Photochem. Photobiol.* 36:721–24
72. Lammers, P. J., Sanders-Loehr, J. 1982. *J. Bacteriol.* 151:288–94
73. Larsen, H., Yocum, C. S., van Niel, C. B. 1952. *J. Gen. Physiol.* 36:161–71
74. Lee-Kaden, J., Simonis, W. 1979. *Plant Cell Physiol.* 20:1179–90

75. Lee-Kaden, J., Simonis, W. 1982. *J. Bacteriol.* 151:229–36
76. Lehman, J. T. 1978. *J. Phycol.* 14:33–42
77. Lockau, W., Pfeffer, S. 1982. *Z. Naturforsch.* 37C:658–64
78. Lockau, W., Pfeffer, S. 1983. *Biochim. Biophys. Acta* 733:124–32
79. Lolkema, J. S., Abbing, A., Hellingwerf, J. K., Konings, W. N. 1983. *Eur. J. Biochem.* 130:287–92
80. Lorrimer, G. H., Osmond, C. B., Akazawa, T., Asami, S. 1978. *Arch. Biochem. Biophys.* 185:49–56
81. Lucas, W. J. 1983. *Ann. Rev. Plant Physiol.* 34:71–104
82. Leuking, D., Tokuhisa, D., Sojka, G. 1973. *J. Bacteriol.* 115:897–903
83. Madigan, M. T., Gest, H. 1978. *Arch. Microbiol.* 117:119–22
84. Marcus, Y., Zenvirth, D., Harel, E., Kaplan, A. 1982. *Plant Physiol.* 69:1008–12
85. Marcus, Y., Harel, E., Kaplan, A. 1983. *Plant Physiol.* 71:208–10
86. Marrs, B. L. 1978. *Curr. Top. Bioenerg.* 8:261–96
87. Michels, P. A. M., Konings, W. N. 1978. *Eur. J. Biochem.* 85:147–55
88. Michels, P. A. M., Hellingwerf, K. J., Lolkema, J. S., Friedberg, I., Konings, W. N. 1981. *Arch. Microbiol.* 130:357–61
89. Miller, A. G., Cheng, K. H., Colman, B. 1971. *J. Phycol.* 7:79–100
90. Miller, A. G., Colman, B. 1980. *Plant Physiol.* 65:397–402
91. Miller, A. G., Colman, B. 1980. *J. Bacteriol.* 143:1253–59
92. Mitchell, P. 1962. *Biochem. Soc. Symp.* 22:142–68
93. More, J. E., Smith, A. J. 1982. *See Ref.* 56, p. B48
94. Murata, N., Sato, N., Omata, T., Kuwabara, T. 1981. *Plant Cell Physiol.* 22:855–66
95. Murata, N., Omata, T. 1982. *See Ref.* 56, p. C20
96. Neilson, A. H., Doudoroff, M. 1973. *Arch. Mikrobiol.* 89:15–22
97. Nielson, A. M., Sojka, G. A. 1979. *Arch. Microbiol.* 120:39–42
98. Nierzwicki-Bauer, S. A., Balkwill, D. L., Stevens, S. E. Jr. 1983. *J. Cell. Biol.* 97:713–22
99. Ohmori, M., Ohmori, K., Strotmann, H. 1977. *Arch. Microbiol.* 114:225–29
100. Olson, J. M. 1980. *Biochim. Biophys. Acta* 594:33–51

100a. Omata, T., Murata, N. 1983. *Plant & Cell Physiol.* 24:1101–12

101. Padan, E., Schuldiner, S. 1978. *J. Biol. Chem.* 253:3281–86
102. Padan, E., Zilberstein, D., Schuldiner, S. 1981. *Biochim. Biophys. Acta* 650: 151–66
103. Parks, L. C., Niederman, R. A. 1978. *Biochim. Biophys. Acta* 511:70–82
104. Paschinger, H. 1977. *Arch. Microbiol.* 113:285–91
105. Peck, H. D. Jr., LeGall, J. 1982. *Phil. Trans. R. Soc. London Ser. B* 298:443–66
106. Perlin, D. S., Spanswick, R. M. 1981. *Plant Physiol.* 68:521–26
107. Peschek, G. A., Broda, E. 1973. *Naturwiss.* 60:479–80
108. Peschek, G. A., Muchl, R., Schmetterer, G. 1981. *Curr. Microbiol.* 6:233–37
109. Peschek, G. A., Muchl, R., Kienzl, P. F., Schmetterer, G. 1982. *Biochim. Biophys. Acta* 679:35–43
110. Peschek, G. A. 1983. *J. Bacteriol.* 153:539–42
111. Pettitt, C. A., Davidson, V. L., Cobb, A., Knaff, D. B. 1982. *Arch. Biochem. Biophys.* 216:306–13
112. Pfennig, N. 1967. *Ann. Rev. Microbiol.* 21:285–324
113. Pfennig, N. 1977. *Ann. Rev. Microbiol.* 31:275–90
114. Pfennig, N. 1978. *The Photosynthetic Bacteria* ed. R. K. Clayton, W. R. Sistrom, pp. 3–18. Plenum New York:
115. Pike, L., Sojka, G. A. 1975. *J. Bacteriol.* 124:1101–6
116. Pinevich, A. V. 1978. *Microbiology* 47:381–87
117. Raboy, B., Padan, E. 1978. *J. Biol. Chem.* 253:3287–91
118. Ramos, J. L., Guerrero, M. G., Losada, M. 1982. *Appl. Envir. Microbiol.* 44: 1020–25
119. Raven, J. A. 1980. *Adv. Microbial Physiol.* 21:48–226
120. Reed, R. H., Rowell, P., Stewart, W. P. D. 1981. *Eur. J. Biochem.* 116:323–30
121. Remsen, C. C. 1978. See Ref. 114, pp. 31–60
122. Resch, C. M., Gibson, J. 1983. *J. Bacteriol.* 155:345–50
123. Revsbech, N.-P., Jørgensen, B. B., Brix, O. 1981. *Limnol. Oceanogr.* 26: 717–30
124. Richey, D. P., Lin, E. C. C. 1972. *J. Bacteriol.* 112:784–790
125. Rippka, R., Waterbury, J. B. 1977. *FEMS Microbiol. Lett.* 2:83–86
126. Rottenberg, H. 1979. *Methods in Enzymol.* LV:547–68
127. Sadler, W. R., Stanier, R. Y. 1960. *Proc. Natl. Acad. Sci. USA* 46:1328–34
128. Saier, M. H., Feucht, B. U., Roseman, S. 1971. *J. Biol. Chem.* 246:7819–21
129. Scherer, S., Sturzl, E., Böger, P. 1981. *Z. Naturforsch.* 36C:1036–40

130. Scherer, S., Böger, P. 1982. *Arch. Microbiol.* 132:329–32
131. Scholes, P., Mitchell, P., Moyle, J. 1969. *Eur. J. Biochem.* 8:450–54
132. Shepherd, W. D., Kaplan, S., Park, J. T. 1981. *J. Bacteriol.* 47:354–62
133. Sherman, L., van de Putte, P. 1982. *J. Bacteriol.* 150:410–13
134. Simpson, F. B., Neilands, J. B. 1976. *J. Phycol.* 12:44–48
135. Singer, R. A., Doolittle, W. F. 1975. *J. Bacteriol.* 124:810–14
136. Sistrom, W. R. 1960. *J. Gen. Microbiol.* 22:778–85
137. Sistrom, W. R. 1977. *J. Bacteriol.* 131: 526–32
138. Smith, A. J., London, J., Stanier, R. Y. 1967. *J. Bacteriol.* 94:972–83
139. Smith, A. J., Hoare, D. S., 1977. *Bacteriol. Rev.* 41:419–48
140. Staehelin, L. A., Golecki, J. R., Drews, G. 1980. *Biochim. Biophys. Acta* 589: 30–45
141. Stanier, R. Y., Cohen-Bazire, G. 1977. *Ann. Rev. Microbiol.* 31:225–74
142. Stanier, R. Y., Pfennig, N., Trüper, H. G. 1981. *The Prokaryotes* ed. M. P. Starr, A. Balows, H. G. Schlegel, pp. 197–211. Berlin: Springer
143. Stewart, W. P. D., Rowell, P. 1975. *Biochem. Biophys. Res. Commun.* 65: 846–56
144. Stewart, W. P. D. 1980. *Ann. Rev. Microbiol.* 34:497–536
145. Stock, J. B., Rauch, B., Roseman, S. 1977. *J. Biol. Chem.* 252:7850–61
146. Storrø, I., McFadden, B. A. 1981. *Arch. Microbiol.* 129:317–20
147. Trüper, H. G. 1978. See Ref. 14, pp. 677–90
148. Trüper, H. G., Fischer, U. 1982. *Phil. Trans. R. Soc. London Ser.* B 298:529–42
149. Uffen, R. L. 1973. *J. Bacteriol.* 116: 874–84
150. Utkilen, H. C., Heldal, M., Knutsen, G. 1976. *Physiol. Plantarum* 38:217–20
151. Utkilen, H. C. 1982. *J. Gen. Microbiol.* 128:1849–62
152. Utkilen, H. C. 1983. *J. Gen. Microbiol.* 129:1713–15
153. van Niel, C. B. 1932. *Arch. Mikrobiol.* 3:1–112
154. Wagner, B. J., Miovic, M. L., Gibson, J. 1967. *Arch. Mikrobiol.* 55:104–12
155. Walsby, A. E. 1982. See Ref. 38, pp. 237–62
156. Yoch, D. C., Zhang, Z., Claybrook, D. L. 1983. *Arch. Microbiol.* 134:45–48
157. Youvan, D. C., Elder, J. T., Sandlin, D. E., Zsebo, K., Alder, D. P., et al. 1982. *J. Mol. Biol.* 162:17–41
158. Zdrou, I., Tromballa, H. W. 1981. *Arch. Microbiol.* 129:325030
159. Zevenboom, W., Mur, L. R. 1981. *Arch. Microbiol.* 129:61–66

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ECOLOGY OF SPIROCHETES

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INTRODUCTION

Spirochetes are a group of helical, motile bacteria that are widely distributed in nature. Many of them exist as free-living forms in the sediments, muds, and surface waters of freshwater ponds, lakes, and streams, in salt marsh sediments, in saline solar lakes, and in deep-sea hydrothermal vents (36, 84, 91). Other spirochetes are found associated with an amazing array of eukaryotic

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hosts, from protozoa to termites to molluscs to mammals (22, 94, 167). Although the overwhelming majority of relationships between spirochetes and their hosts are benign and mutualistic, some spirochetes are pathogenic to animals and humans, causing syphilis, relapsing fever, swine dysentery, Lyme disease, leptospirosis, and other diseases (15, 78, 184).

Spirochetes (order *Spirochaetales*) as a taxon have been defined on the basis of their characteristic morphology and arrangement of cellular components (28). All spirochetes have a helical cell body or "protoplasmic cylinder" consisting of cytoplasmic and nuclear regions enveloped by a cell membrane and cell wall. The helical shape of spirochete cells is maintained by their peptidoglycan layer (107, 108). The protoplasmic cylinder is wound together with filamentous structures called "axial fibrils," "periplasmic fibrils," "flagella," "endoflagella," or "periplasmic flagella" (Figure 1). The number of these fibrils present per cell ranges from 2 to more than 100, depending on the kind of spirochete. One end of each periplasmic fibril is inserted near one pole of the protoplasmic cylinder, whereas the other end is not inserted. Approximately equal numbers of fibrils are anchored at opposite ends of the protoplasmic cylinder and may or may not overlap in the center portion of the cell, depending on the kind of spirochete. The protoplasmic cylinder and attached periplasmic fibrils are completely surrounded by an outer membranous structure called the "outer sheath" or "outer cell envelope" (Figure 1). This structure corresponds to the outer membrane of other gram-negative bacteria (94). The periplasmic fibrils and the basal bodies that anchor them are structurally and chemically similar to bacterial flagellar assemblies (35, 94), and it is now known that these fibrils are the organelles of motility in spirochetes (26, 147). However, because the periplasmic fibrils are completely surrounded by the outer sheath, they are not in direct contact with the external environment. Thus, the mechanism of spirochete motility must be, in some ways, fundamentally different from the motility mechanism of flagellated bacteria.

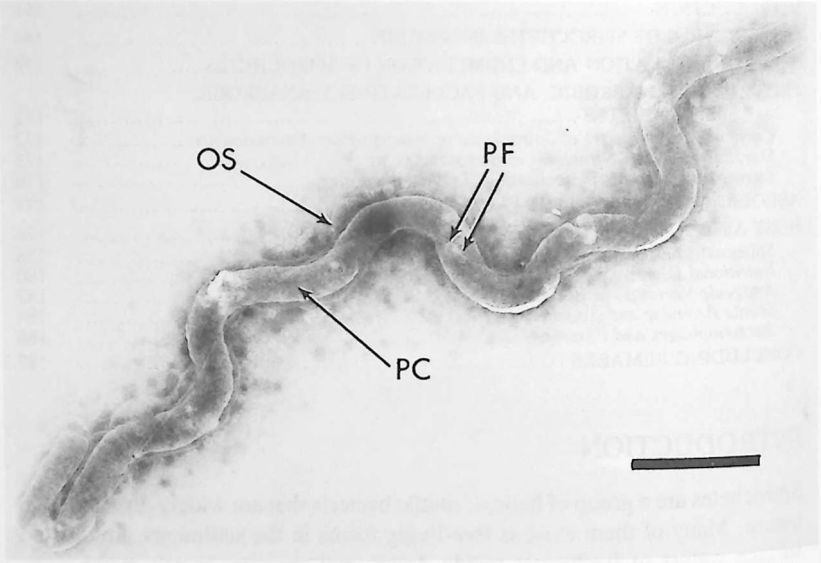


Figure 1 An electron micrograph of a negatively stained cell of an anaerobic marine spirochete. The periplasmic fibrils *(PF)* are wound around the protoplasmic cylinder *(PC)*. An outer sheath *(OS)* surrounds both the protoplasmic cylinder and periplasmic fibrils. Bar = 1 μm.

Among bacteria only the spirochetes possess endocellular organelles of motility wound around a helical cell body. Probably because of this unique morphological feature, the spirochete cell exhibits a motility behavior that is not observed in other groups of bacteria. Spirochetes perform rotatory, locomotory, and flexing movements when they swim in liquid environments (35). Furthermore, spirochetes move through environments of viscosity high enough to impede the progress of most flagellated bacteria (16, 71, 72, 151). In addition, spirochetes move on solid surfaces by "creeping" or "crawling" (18, 48), a type of locomotion similar to that of gliding bacteria.

Even though all spirochetes share common morphological and motility characteristics, they are an extremely physiologically diverse group of bacteria. For example, aerobic, facultatively anaerobic, and obligately anaerobic spirochetes have been described (28). Within these groupings are represented spirochetes with a remarkable diversity of nutritional requirements and energy-yielding mechanisms. For example, certain rumen and oral spirochetes derive energy almost exclusively from the fermentation of plant polymer materials (201, 209), while others ferment a wide variety of sugars and amino acids (93). Other spirochetes dissimilate only certain long-chain fatty acids (106).

Some spirochetes grow well in mineral media supplemented with a single carbon and energy source, whereas others require complex medium constituents such as calf serum. Many other spirochetes have eluded all attempts at cultivation (34).

The guanine-plus-cytosine (G + C) content of the DNA of spirochetes ranges from 25 to 66 mol% (28, 74, 136, 138). Thus, analysis of DNA base composition supports the conclusion, based on physiological studies, that spirochetes are a phylogenetically diverse group of bacteria. Oligonucleotide cataloging of 16S ribosomal RNA has reinforced this concept (63, 149).

The ability of any bacterial species or group to persist in its physical environment and to compete successfully with other organisms for available nutrients results from complex physiological and behavioral adaptations. In this review we describe physiological and behavioral adaptations of spirochetes that apparently endow these bacteria (or some of these bacteria) with the ability to compete successfully under particular environmental conditions. We have

included published material that deals primarily with the physiology of spirochetes but has direct bearing on the ecology of these bacteria.

In addition, we summarize the current knowledge of the distribution of spirochetes in nature and we discuss studies in which spirochetes present in various habitats have been enumerated. Our discussion is focused on those aspects of spirochete ecology and physiology that have been studied more extensively. Thus, it is not our intent to present a comprehensive review of spirochete ecology but rather to concentrate on selected topics. At the same time we attempt to draw attention to areas that, in our opinion, deserve further study.

DISTRIBUTION OF SPIROCHETES IN NATURE

Ecological as well as morphological and physiological criteria have been used to classify spirochetes into five genera: *Leptospira, Spirochaeta, Cristispira, Treponema,* and *Borrelia* (28). Natural distribution is a heavily weighted component of spirochete taxonomy. Thus, it seems appropriate to accompany a discussion of the distribution of spirochetes in nature with a summary of the present classification of these bacteria. The distribution of various spirochetes that have not been classified is also reviewed.

Members of the genus *Leptospira* are obligately aerobic spirochetes that have slender, tightly coiled cells and a distinctive motility behavior that readily distinguishes them from other spirochetes (48). Some *Leptospira* strains are saprophytes, found free-living in water and soil (the "biflexa complex"), whereas others are pathogens that infect mammals (the "interrogans complex") (101). Pathogenic leptospires are also found, at times, in water and soil environments (2, 88). Members of the genus are distinguished on the basis of antigenic characteristics and are subdivided into serovars or serogroups (52, 54). Pathogenic leptospires are the causative agents of leptospirosis, a common and important disease of livestock. The interrogans complex has an exceedingly broad host range. Members of this group infect not only domestic animals such as dogs, cattle, and swine, but they also have been isolated from many kinds of wild animals, including rodents, skunks, raccoons, foxes, opossums, and sea lions (75).

When present in hosts, pathogenic leptospires reside and multiply in the convoluted tubules of the mammalian kidney (2). Leptospires are shed in the urine of infected animals, and long-term (more than one year) excretion occurs in several wildlife species, such as rats and raccoons, that serve as natural reservoirs for the dissemination of leptospirosis (75). The duration of leptospire infections varies depending on the host and the serovar causing the infection (2). Bodies of water contaminated with the urine of infected animals serve as sources of pathogenic leptospires, and transmission of members of the interorgans complex to new hosts often occurs during contact with contaminated surface water (2). In neutral or slightly alkaline waters, pathogenic leptospires survive for three months or longer (2).

Thus, the natural distribution of pathogenic leptospires is related not only to their persistence in reservoir animals, but also to their ability to survive as free-living forms in aquatic environments. Humans may acquire leptospirosis through exposure to environments contaminated by pathogenic leptospires or through contact with urine, blood, or tissues of infected animals (184). Usually humans represent dead ends in the chain of infection, as transmission of the disease from one person to another is rare (184).

Members of the "biflexa complex," also referred to as saprophytic leptospires or free-living leptospires, are widely distributed in nature and have been isolated from soil and water collected in many different geographical locations (40, 43, 91, 155). Free-living leptospires have been isolated from marine (42) as well as freshwater environments. The marine *Leptospira* require relatively high concentrations of sodium ions for growth and therefore are specifically adapted to life in seawater (41).

Members of the genus *Leptospira* form a cohesive and specialized group in terms of their nutritional requirements. They utilize long-chain fatty acids as major carbon and energy sources for growth (90, 106), and all require fatty acids for lipid synthesis (89, 104). Amino acids or carbohydrates are not utilized as carbon and energy sources. All leptospires are similar in size, with cell diameters approximately 0.1 μm and cell length ranging from 6–20 μm. With the exception of serotype *illini* (mol% G+C = 53) (76), the leptospires form a cluster in terms of the G+C content of their DNA (35.3–41 mol% G+C) (74). Analysis of 16S ribosomal RNA indicates that *Leptospira* strains are phylogenetically remote from other spirochetes, suggesting that leptospires diverged from other spirochetes very early in the evolutionary history of the *Spirochaetales* (63).

Anaerobic and facultatively anaerobic spirochetes that exist exclusively as free-living forms are classified in the genus *Spirochaeta* (mol% G+C = 50–66%). Members of this genus have been observed in and isolated from many different aquatic habitats (36). For example, fresh water strains have been isolated from pond mud and water (24, 37), and salt-requiring strains from intertidal salt-marsh sediments (83, 92) as well as from water surrounding a deep-sea hydrothermal vent (84). A moderately halophilic spirochete has been isolated from mud collected in a high salinity pond (69), and thermophilic spirochetes have been observed in hot water springs (S. H. Zinder, personal communication).

Although members of the genus *Spirochaeta* exist in diverse environments and differ with respect to salt and temperature requirements, those species that have been characterized exhibit marked similarities in growth substrate prefer-

ences and energy-yielding metabolism (34). All species of *Spirochaeta* that have been cultivated and studied use carbohydrates, and essentially no other compounds, as oxidizable substrates for growth (23, 24, 39, 69, 83, 92). None are known to break down cellulose or pectin, and only a few marine strains (83) have been found to degrade hemicellulosic material, such as xylan. However, no systematic studies have been conducted to enumerate plant polysaccharide-utilizing, free-living spirochetes in nature.

Carbohydrates that are utilized as carbon and energy sources by *Spirochaeta* strains are hexoses, disaccharides, and pentoses commonly released from plant polysaccharides (e.g. cellulose, hemicellulose) through the metabolic activities of bacteria other than spirochetes. Because *Spirochaeta* strains usually are present in anaerobic environments rich in decaying plant materials, it may be inferred that, in such environments, spirochetes are active in the fermentation of soluble sugars formed by the enzymatic activities of plant polysaccharide-degrading microorganisms.

Members of the genus *Spirochaeta* measure 0.2−0.5 μm by 5–40 μm, with the exception of *S. plicatilis*. This as yet uncultivated spirochete is exceptionally large, measuring 0.75 μm by 80–250 μm (18).

The genus *Cristispira* includes large (0.5–3.0 μm by 30–180 μm), morphologically distinct (120) spirochetes that inhabit the digestive tract of marine and freshwater molluscs (e.g. clams, mussels, and oysters). These spirochetes have become adapted to life in the specialized habitat of the crystalline style of molluscs (189). The crystalline style is a mucoproteinaceous rod-shaped structure present in the stomach of molluscs, where it functions much like a pestle to grind food particles against the gastric shield (a rigid area present in a portion of the stomach wall). To date no strain of *Cristispira* has been propagated in pure culture. For this reason almost nothing is known about the physiology of these spirochetes. Two review articles have detailed the relationships that exist between *Cristispira* and their molluscan hosts (22, 118).

Anaerobic spirochetes present in a wide spectrum of habitats within mammalian hosts are classified in the genus *Treponema*. Many constitute part of the normal indigenous microbiota present in the mouth, the intestine, and the genital regions of humans and other animals. Treponemes can be routinely isolated from the bovine rumen (148, 176, 208) and are frequently seen when rumen contents are examined under the microscope. Treponemes have been observed in or isolated from the intestinal tracts of apparently healthy or diseased dogs, monkeys, opossums, mice, rats, and humans (79, 122, 139, 161, 162, 187, 188, 193–195). There are reports of a positive correlation between the presence of spirochetes and intestinal disorders in some of these animals and in humans (4, 65, 121, 161). Other investigators maintain, however, that colorectal spirochetosis has no clinical significance (144).

Many strains of treponemes have been isolated from the intestines of swine (49, 79, 152). One of these, *T. hyodysenteriae*, is the causative agent of swine

dysentery and the only pathogenic treponeme that has been cultivated in vitro in pure culture (78). Swine dysentery is a highly infectious disease (171) characterized by profuse bleeding from the rectum and extensive erosion of the mucosal epithelium (68). The disease interferes with the gain of weight by swine and for this reason is the cause of major economic losses in the pork industry.

Although many studies aimed at establishing the etiology of swine dysentery have been conducted, little is known about physiological characteristics of *T. hyodysenteriae* that may be responsible for its virulence. *T. hyodysenteriae* resembles in many respects *T. innocens*, a nonpathogenic species present in the intestine of clinically normal swine. The two spirochetes are similar in morphology and in the G+C contents of their DNAs (117, 138). Both spirochetes also inhabit similar if not identical environments. They have been isolated not only from the intestinal contents and feces of swine, but also from the cecal contents of rodents trapped on farms where swine dysentery had occured (99).

Despite these similarities, *T. innocens* and *T. hyodysenteriae* can be differentiated with respect to lipid composition and β-hemolysin production (117, 135). This raises the possibility that these or other characteristics that distinguish these two spirochete species may play a role as virulence factors in *T. hyodysenteriae*. Ultimately, however, it will be necessary to compare avirulent derivatives of *T. hyodysenteriae* with virulent parent strains in order to establish the biological basis for the pathogenesis of swine dysentery.

Other pathogenic treponemes include *T. pallidum*, *T. carateum*, and *T. pertenue*, the causative agents of syphilis, pinta, and yaws, respectively (205). These treponeme species are morphologically indistinguishable, and Miao & Fieldsteel (137) have shown that *T. pallidum* and *T. pertenue* are genetically indistinguishable. Thus, even though the above-mentioned treponematoses are clinically distinct, the distinction may not be due to differences in the causative agents of the diseases.

T. pallidum (Nichols) has been found to have many of the physiological attributes of an aerobic bacterium (8, 14, 47, 130, 143), although it shows some sensitivity to 0_2 (58). Thus, *T. pallidum* differs from *Treponema* species that have been grown in pure culture, as all cultivated treponemes are obligate anaerobes. *T. pallidum* has been cultivated in vitro in tissue culture (57, 58, 98). The pathogenesis and immunology of treponemal infections of humans have been extensively reviewed recently (163).

Avirulent as well as virulent treponemes have been isolated from pathological processes of the human genital region (28). Two of these avirulent treponemes, *T. phagedenis* and *T. refringens*, are genetically distinct from each other as well as from *T. pallidum* (136, 137). *T. phagedenis* (the Reiter treponeme) has been extensively characterized in terms of its growth requirements and energy-yielding metabolism (3, 66, 67).

Many strains of oral treponemes have been isolated from subgingival plaque and the gingival crevice area of humans (126, 170, 201). Several studies suggest a possible relationship between the presence of large numbers of spirochetes and periodontal disease in humans (5, 97, 127, 129, 185). The genus *Treponema* is a genetically diverse taxon with G + C contents ranging from 25.8 to 53.7 mol% (136, 138). Treponemes range in size from 0.2–0.7 μm by 5–20 μm.

The genus *Borrelia* consists of arthropod-borne spirochetes that have adapted to a rather complex set of ecological circumstances. Borreliae are transmitted by ticks or lice to vertebrate hosts (e.g. humans, birds, and rodents) and cause diseases such as relapsing fevers. The etiologic agents of louse-borne and tick-borne relapsing fevers in humans are *B. recurrentis* and *B. duttoni*, respectively (30, 55, 116).

Borrelia species that apparently are distinct from those infecting humans and rodents infect many different kinds of birds. One species, *B. anserina*, is the etiological agent of avian spirochaetosis, a highly fatal disease of poultry (116). The natural distribution of any given species of *Borrelia* is intimately connected with the biology of its arthropod vector (29, 55). In fact, borreliae are classified into species according to the species of arthropod that transmits them (28). However, this classification scheme probably will be revised, as it is known now that a given *Borrelia* strain is often capable of infecting more than one tick species.

After lice or ticks feed on a vertebrate host infected with *Borrelia*, the spirochetes penetrate the gut wall of the arthropod and invade the hemolymph of the coelomic cavity. The *Borrelia* multiply in the coelomic cavity and from this point can disseminate to other tissues. In lice, the tissues of the genital region are not invaded, and for this reason the spirochetes are not vertically transmitted to louse progeny (29, 116). The reproductive tissues of ticks, on the other hand, can be invaded by *Borrelia*, and studies show that the spirochetes are often transovarilly transmitted to tick larvae (29, 64, 207). Individual ticks have been reported to remain viable and infectious for up to twelve years (55, 116), making these arthropods suitable hosts for the maintenance of *Borrelia* in the laboratory.

Tick-borne borreliae are transmitted to the blood of their vertebrate host either directly, through saliva during biting, or via infectious body fluids (coxal fluid) (30). Noninfected ticks can become infected with *Borrelia* by feeding on rodent hosts, suggesting that rodents serve as natural reservoirs for borreliae (116). Humans become infected with louse-borne relapsing fever borreliae (*B. recurrentis*) by contamination of the bite wound with hemolymph of infected lice that have been crushed during scratching. Borreliae released from the crushed lice penetrate the body through the bite wound or through skin (30).

A few *Borrelia* strains have been grown in vitro in pure culture (116, 182).

However, cell yields are typically very low, and to date very little is known about the physiology and genetics of these bacteria. Borreliae have the interesting property of being able to undergo antigenic variation. This capability, which will be discussed later in this review, may have relevance in terms of the ecological success of this group of spirochetes. The G+C content of *Borrelia* DNA is unknown. Borreliae have loosely coiled cells and measure 0.2–0.5 μm in diameter by 5–25 μm in length (30).

Spirochetes that are quite distinct morphologically from *Borrelia* have been isolated from *Ixodes ricinus* and *Ixodes dammini*, two tick species that have been implicated as vectors for the transmission of erythema chronicum migrans (ECM) and Lyme disease, respectively (31, 32, 180). ECM is a syndrome characterized by a distinctive skin lesion, and Lyme disease is a form of inflammatory arthritis. That the *I. dammini* spirochete (IDS) is the causative agent of Lyme disease has been firmly established by investigators who isolated the spirochete from the blood, skin, and cerebrospinal fluid of patients afflicted with this disorder (15, 179).

One early manifestation of Lyme disease is the occurence of a skin lesion similar to those seen in cases of ECM (110, 181). Investigators have noted that the *I. ricinus* and *I. dammini* spirochetes are similar in morphology, whole-cell polyacrylamide electrophoresis patterns, and antigenic determinants. Thus, it seems likely that ECM and Lyme disease represent two different expressions of the same infection process (9, 32). In common with *Borrelia*, the natural distribution of the *I. dammini* spirochete is closely associated with the life cycle of its tick vector. The spirochete has been isolated from white-footed mice and from white-tailed deer, two hosts of *I. dammini*. The white-tailed deer serves as host for representatives of each stage of the *Ixodes* tick life cycle, a fact that suggests that this mammal serves as a natural reservoir for the spirochete (21).

The taxonomic relationship between *Borrelia* and the ixoid tick spirochetes remains unclear. The *I. dammini* and *I. ricinus* spirochetes are tick-associated and readily grown in media suitable for cultivation of *Borrelia*. However, they have tightly coiled cell morphologies that are not typical of *Borrelia* and are more reminiscent of the cell morphologies of some of the treponemes (9, 31). At this writing almost nothing is known about the physiology of the ixoid tick spirochetes, and relatively little work has been done on *Borrelia* physiology. Clearly, more research is required to establish a relationship between these two pathogenic forms of tick-associated spirochetes.

Numerous other host-associated spirochetes have been described but, for various reasons, have not yet been classified according to recognized taxonomic criteria. These include spirochetes associated with the gut of termites and wood-eating cockroaches (22, 132, 192), the mesogleal region of *Hydra attenuata* (96), planorbid molluscs (154), and the tissues of the brine shrimp, *Artemia salina* (196). Spirochetes occur as free-living forms in activated

sewage sludge (51) and in sewage present in thermophilic digestors (S. H. Zinder, personal communication). It is possible that the spirochetes observed in sewage are, in fact, host-associated forms that were shed by their hosts.

SELECTIVE ISOLATION AND ENUMERATION OF SPIROCHETES

Various selective enrichment procedures have been used for the isolation of spirochetes (36, 101, 116, 167). One method is based on the ability of thin spirochetes to pass through membrane filters of pore diameters small enough (0.22–0.45 μm) to retain most other kinds of bacteria. This procedure places a size limitation on the spirochetes that are isolated, inasmuch as their cells usually have a diameter of less than 0.5 μm.

Another method relies on the ability of spirochetes to migrate through agar media and away from contaminating bacteria. Spirochetes differ from most flagellated bacteria in that they have the ability to move through media containing as much as 1.0–2.0% (wt vol^{-1}) agar. Localized bacterial growth (e.g. colonies) on agar media generates a concentration gradient of the carbon and energy source utilized by the cells. Because spirochetes are chemotactic and are able to move through agar media, they migrate toward the higher concentrations of the gradient (24). This behavior results in the formation of a growth "veil" or "ring" that gradually moves outward towards the periphery of agar medium plates and away from contaminating organisms that may be present in the original area of growth. In isolation procedures, spirochetes from the outermost edge of such veils are streaked on agar medium plates or diluted through tubes of agar medium to obtain pure cultures.

Enrichment procedures that take advantage of the ability of spirochetes to migrate through agar gels or to pass through the pores of membrane filters have been used to isolate many strains of *Leptospira, Spirochaeta,* and *Treponema. Borrelia* have been isolated by direct inoculation of media with the blood of mice heavily infected with these spirochetes (116). Infection of animals with *Borrelia* may therefore be viewed as a kind of selective enrichment procedure.

Selective media for the direct isolation of spirochetes have been developed. For example, *T. hyodysenteriae* has been selectively isolated under anaerobic conditions on plates of medium containing the antibiotic spectinomycin (172). The pyrimidine analog 5-fluorouracil has been used as a selective agent for the isolation of leptospires (91, 105).

More recently, a procedure involving the antibiotic rifampin (rifampicin) has been developed and used for the selective isolation of spirochetes from the human gingival crevice region, the bovine rumen, freshwater ponds, intertidal marine mud, and deep-sea water (84, 125, 126, 176, 203). The inhibitory effect of rifampin on many bacteria other than spirochetes markedly increases

the numerical ratio of spirochete colonies to other bacterial colonies present in agar media. Spirochete colonies are distinguishable from other bacterial colonies on the basis of their appearance (148) and thus can be enumerated readily in selective agar media inoculated with materials containing mixed bacterial populations. Both free-living and host-associated spirochetes isolated from nature by methods that did not involve the use of rifampin were found to be resistant to relatively high concentrations of this antibiotic (125, 126, 176).

This observation suggests that resistance to rifampin may be a general property of spirochetes. In vitro RNA polymerase activity of *S. aurantia* was not appreciably affected by a concentration of rifampin that completely inhibited *Escherichia coli* RNA polymerase. This and other experiments support the idea that rifampin resistance is due to a low affinity of spirochete RNA polymerase for this antibiotic (125). An important advantage of isolation procedures involving rifampin-containing media is that they can be used to isolate large spirochetes (e.g. with cell diameters greater than 0.5 μm) (176). Thus, the rifampin method avoids size restrictions imposed by enrichment methods based on filtration.

Direct isolation procedures involving rifampin as a selective agent have allowed investigators to enumerate spirochetes present in environments such as a salt marsh and the bovine rumen, and to assess the extent of their physiological diversity (148, 176, 203). Weber & Greenberg (203) enumerated anaerobic spirochetes present in various salt-marsh habitats by diluting samples of sediment into tubes of a complex agar medium containing cellobiose as the major fermentable carbohydrate. In the absence of the selective agent (rifampin), approximately 7% of the colonies that developed in this medium had the distinctive appearance of spirochete colonies. In the presence of rifampin, the numerical ratio of spirochete colonies to other bacterial colonies increased. The higher numerical ratio enabled the investigators to enumerate with accuracy the spirochetes present in sediments. A gram (wet weight) of the upper 1 cm of anoxic sediment was found to contain 10^4–10^6 spirochetes, depending on the salt-marsh habitat. Apparently, salt marsh sediments provide favorable habitats for spirochetes.

Anaerobic spirochetes isolated from marine muds or sediments are carbohydrate-fermenters and do not use other compounds as fermentable substrates for growth (83, 92). Attempts to isolate marine, amino-acid fermenting spirochetes have not succeeded (83). Thus, it is likely that the salt-marsh spirochetes that formed colonies in Weber & Greenberg's (203) agar medium utilized the cellobiose present in the medium as the major fermentable substrate. The presence of high numbers of carbohydrate-fermenting spirochetes in salt-marsh sediments supports the idea that free-living spirochetes present in anaerobic environments function primarily in the breakdown of sugars released from decaying plant material.

Studies on the enumeration and diversity of rumen spirochetes (148, 176, 177) are discussed below.

FREE-LIVING, ANAEROBIC, AND FACULTATIVELY ANAEROBIC SPIROCHETES

Chemotactic Responses of Spirochetes in Nutrient-Poor Environments

Bacteria are routinely exposed to conditions of nutrient insufficiency in the environments that they inhabit (190). As pointed out by Veldkamp (199), "nutrient limitation is the rule rather than the exception" in natural ecosystems. When nutrients essential for growth are present at very low concentrations, bacteria express physiological processes different from those that function in the presence of excess nutrients (77, 190). To gain an understanding of the physiology of bacteria as they exist under the prevailing conditions in their habitats, it is useful to study bacterial cells grown in vitro under nutrient-limited conditions.

Studies on the chemotactic behavior of spirochetes grown in continuous culture have shown that at least some of these bacteria regulate their chemosensory mechanisms in response to growth substrate limitation (191). The spirochete used in these studies was *S. aurantia* M1, a saccharolytic facultative anaerobe that is chemotactic toward numerous carbohydrates (70). *S. aurantia* was grown in a chemostat under conditions of energy and carbon source limitation at different dilution rates. The dilution rate (D) is defined as the flow rate per unit volume (153). An effect of varying D is to change the steady-state, growth-limiting concentration of nutrients in the chemostat culture.

Thus, a decrease in the dilution rate results in a decrease in the steady-state concentration of the carbon and energy source utilized for growth by the microorganisms in the culture vessel (134). The chemotactic responses of *S. aurantia* cell populations grown at different D values were measured, as was the response of cells grown in batch culture (in the presence of excess carbon and energy source). Measurements of chemotactic response were carried out by determining the number of cells that accumulated in a capillary tube containing a solution of attractant (70).

When glucose was the limiting energy and carbon source in the growth medium, the greatest chemotactic response toward this sugar was exhibited by cells grown at $D = 0.045\ h^{-1}$, a dilution rate at which the growth-limiting glucose concentration in the culture was extremely low. Cells grown at higher D values (higher glucose concentrations) had markedly lower chemotactic responses. When the cells were grown at D values lower than $0.045\ h^{-1}$, their response to glucose declined, apparently because they could not maintain a fully functional chemotactic system under the near-starvation culture conditions. The results indicated that *S. aurantia* cells expressed their maximum chemotactic response at a distinct dilution rate, which was termed "peak D" (191). The chemotactic response of cells grown in the medium containing nonlimiting concentrations of glucose (batch culture) was much smaller than that of chemostat-grown (peak D) cells.

Furthermore, the chemotactic response toward glucose was specifically enhanced when this sugar was the growth-limiting energy and carbon source, and was not enhanced when the growth-limiting energy and carbon source was xylose, which is recognized by a different chemoreceptor (191; Terracciano & Canale-Parola, unpublished results). Likewise, when *S. aurantia* cells were grown in a chemostat under xylose limitation, their chemotactic response to xylose was enhanced, as compared to cells grown under glucose limitation.

It was also found that *S. aurantia* cells grown in the chemostat at peak D detected lower attractant (glucose) concentrations than cells grown at higher D values or in batch culture. Other experiments excluded the possibility that the enhancement of glucose chemotaxis was due to selection for "super-chemotactic" mutants when cells were grown under glucose limitation in the chemostat, or that different rates of glucose utilization present in cells grown at different D values affected the determinations of chemotactic responses (Terracciano & Canale-Parola, unpublished data).

This work demonstrates that growth at low substrate concentrations elicits an enhanced chemotactic response in *S. aurantia*. Thus, this spirochete possesses regulatory mechanisms that are advantageous under conditions of severe nutrient limitation. It is likely that, under conditions of nutrient deficiency that are common in natural environments, such regulatory mechanisms equip the spirochetes with important selective advantages.

The processes responsible for regulation of the chemosensory apparatus in cells grown at limiting substrate concentrations have not been investigated at the molecular level. In these cells, regulatory processes may operate that affect the number of chemoreceptor molecules per cell, the nature of the chemoreceptor molecule, the receptor-attractant affinity, and/or the methylation of transducer proteins (87, 113, 131).

Starvation-Survival Strategies of Spirochaeta

Depletion of nutrients in natural environments frequently occurs as a consequence of the metabolic activities of indigenous microorganisms (77). This is illustrated by the nutritional circumstances of a salt marsh, an environment that supports the growth of heterotrophic microorganisms to high population densities. In such an environment there is intense competition among bacteria for growth substrates (e.g. soluble sugars) released from polysaccharides and other polymers present in decaying plant material by the enzymatic activities of specialized groups of microorganisms.

As a result of this competition, bacteria unable to degrade polysaccharides and other plant polymers are likely to be confronted, at times, with periods of complete carbon and energy source deprivation. These conditions of nutrient starvation are fundamentally different from situations. such as those described in the preceding section, where nutrients are present at very low concentrations (50).

Spirochetes have relatively slow growth rates, are not known to form endogenous energy storage compounds, and many of them are quite specialized with respect to the kinds of compounds that they use as carbon and energy sources. Nevertheless, spirochetes persist in anaerobic sediments and compete effectively with other heterotrophic microorganisms for soluble sugars that become available. Work on the obligately anaerobic spirochete *S. isovalerica* has provided insights into physiological strategies used by at least some spirochetes to survive and compete with other bacteria during periods of starvation resulting from the depletion of growth substrates in natural environments (80–82).

S. isovalerica, like other species of *Spirochaeta,* utilizes carbohydrates, but not amino acids, as fermentable substrates for growth. This organism has, however, the ability to dissimilate small amounts of certain exogenously supplied amino acids with formation of characteristic fermentation products. *S. isovalerica* ferments the branched-chain amino acids L-leucine, L-isoleucine, and L-valine, forming the branched-chain fatty acids isovalerate, 2-methylbutyrate, or isobutyrate, respectively, as fermentation products that are excreted into the growth medium. Although *S. isovalerica* ferments these amino acids, they do not serve as sole carbon and energy sources for growth when supplied either individually or in combinations to the spirochete.

Harwood & Canale-Parola (80) found that, in the absence of a fermentable substrate (e.g. glucose), the survival of *S. isovalerica* was prolonged when the cells were suspended in a starvation buffer solution containing the three branched-chain amino acids mentioned above, as compared to survival in buffer solution lacking the amino acids. The starving spirochetes fermented the branched-chain amino acids, as indicated by the finding that isovalerate, 2-methylbutyrate, and isobutyrate accumulated in the buffer solution. A separate study, carried out to elucidate the metabolic pathways utilized by *S. isovalerica* to ferment leucine, isoleucine, and valine, showed that the fermentation of branched-chain amino acids by *S. isovalerica* yielded ATP via a substrate-level phosphorylation step (81).

This finding indicated that cell populations starved in the presence of branched-chain amino acids generated ATP. Apparently this ATP was used as a source of maintenance energy to prolong cell survival by sustaining growth-unrelated cell functions (e.g. maintenance of the electrochemical gradient and turnover of macromolecules) during starvation. In their natural habitat the spirochetes may catabolize branched-chain amino acids as a survival strategy when growth substrates are not available. Proteases excreted by *S. isovalerica* (80) probably function to increase the concentration of branched-chain amino acids available in natural environments. In addition, endocellular protein may be used by the spirochetes as a source of branched-chain amino acids.

The ATP-yielding step in the fermentation of leucine, isoleucine, and valine by *S. isovalerica* is catalyzed by a branched-chain fatty acid kinase (82). This kinase functions exclusively in the generation of branched-chain fatty acids and ATP during branched-chain amino acid metabolism. It does not catalyze the formation of fatty acids (e.g. acetate) that are end products of carbohydrate fermentation by the spirochete. These findings indicate that physiological mechanisms have evolved in *S. isovalerica* that are used specifically for the generation of maintenance energy from amino acids during periods of carbohydrate starvation.

At present it is not clear how widespread among spirochetes is the ability to dissimilate minor amounts of amino acids for purposes of prolonging survival. In a survey of 14 independently isolated strains of anaerobic marine spirochetes, 6 had the ability to ferment leucine, isoleucine, and valine (80). Therefore, branched-chain amino acid fermentation is not an uncommon characteristic among marine spirochetes.

The cells of many bacterial species break down endogenous ribosomal RNA when exogenous carbon and energy sources are not available (50). It has been suggested that metabolic intermediates (such as ribose, purine, and pyrimidine bases) formed from the breakdown of endogenous RNA are metabolized further to generate ATP, which may be used in cell maintenance processes by starving bacteria (73). However, specific enzymatic steps for this type of ATP generation have not been demonstrated in starving cells. In addition to deriving energy from catabolism of endogenous RNA, starving bacteria may generate ATP by breaking down endogenous protein.

There are indications that these types of starvation processes are utilized by spirochetes. Preliminary work indicates that the cellular RNA content of free-living spirochetes decreases significantly when they are starved in the absence of carbon and energy sources (Terracciano & Canale-Parola, unpublished data). Furthermore, anaerobic spirochetes (e.g. *S. stenostrepta*) possess enzymes that catalyze the formation of ribose-1-phosphate and purine bases from nucleotides that may be derived from RNA breakdown (38). *S. stenostrepta* and other spirochetes utilize D-ribose as an exogenous energy source for growth (24, 83, 92). Thus, these spirochetes possess enzymes needed to catabolize D-ribose and to generate ATP from this pentose.

These observations strongly suggest that *S. stenostrepta* and probably other spirochetes generate ATP during starvation by metabolizing endogenous RNA. In addition, studies of branched-chain amino acid metabolism in *S. isovalerica*

showed that these spirochetes have the ability to degrade cellular protein (80). Energy-yielding metabolism of ribosomal RNA and cellular protein may be important survival processes for spirochetes during starvation, inasmuch as these bacteria are not known to form endogenous energy storage compounds such as glycogen or poly-β-hydroxybutyrate, and there is no ultrastructural evidence for the formation of any inclusion bodies that may be construed as being stored energy reserves (94).

Interactions Between Spirochaeta *and Other Bacteria*

Spirochetes are frequently observed in anaerobic enrichment cultures for marine cellulolytic bacteria, especially when these cultures are inoculated with marsh sediments. This observation suggests that free-living spirochetes associate and interact with cellulose-degrading bacteria in anaerobic marine environments. It is likely that the spirochetes utilize for growth soluble sugars released from cellulose by anaerobic cellulolytic bacteria, as has been shown for some rumen spirochetes (177).

It is also possible that amino acid–fermenting salt-marsh spirochetes, such as *S. isovalerica,* provide cellulolytic bacteria with branched-chain fatty acids required as growth factors by the latter organisms. This type of interaction would provide both the spirochetes and the cellulolytic bacteria with a selective advantage. Similar nutritional interactions have been described for rumen bacteria (27, 140).

Other observations suggest that interactions exist between free-living anaerobic spirochetes and photosynthetic bacteria. Veldkamp (198) described a saccharolytic anaerobic spirochete *(S. zulzerae)* that occurred in a liquid enrichment culture for green photosynthetic bacteria that was inoculated with mud from a freshwater pond and incubated in the light.

Spirochetes have also been observed in marine enrichments for photosynthetic bacteria. These anaerobic enrichment cultures were prepared in mineral medium solidified with agar and inoculated by stabbing marine marsh mud with a needle through the center of the tube of medium. During incubation in the light, growth of green photosynthetic bacteria occurred along the stab where the mud had been inoculated. Fluffy white colonies of spirochetes occurred at the periphery of this growth (J. Gibson, personal communication; C. S. Harwood, unpublished observations). The nature of the association between spirochetes and photosynthetic bacteria is not known. However, since the marine enrichment culture mentioned above contained only agar and CO_2 as carbon sources, it is speculated that the spirochetes utilized, as growth substrates, carbon compounds excreted by the photosynthetic bacteria (166).

It has been reported that an interaction occurs between the large, as yet uncultivated spirochete, *S. plicatilis,* and the sulfur-oxidizing bacterium, *Beggiatoa. S. plicatilis* cells were observed within mats of sulfur-containing

Beggiatoa trichomes present in samples of sulfide-rich mud collected from a brackish marsh (18). The *Beggiatoa* mats developed near the interface between the mud and a layer of water that covered it, during the first weeks after collection. The large spirochetes swam within the tangled masses of *Beggiatoa* trichomes and were also seen "creeping" on the surfaces of the trichomes.

In time, as the sulfide level in the mud became low, the *Beggiatoa* population gradually lysed and, simultaneously, the number of large spirochetes increased dramatically. The spirochetes accumulated near the portions of the *Beggiatoa* trichomes that were lysing, possibly because they were attracted chemotactically to compounds released by the disintegrating trichomes.

AEROBIC SPIROCHETES: THE LEPTOSPIRES

Leptospires are commonly isolated from aerobic oligotrophic environments, including surface waters of lakes and streams that contain chronically low levels of dissolved organic matter (91). This observation, coupled with the fact that leptospires are extremely specialized with respect to the kinds of growth substrates they utilize (101), indicates that members of this genus of spirochetes must have evolved appropriate mechanisms for scavenging nutrients and/or for surviving during long periods of starvation.

In oligotrophic aquatic environments, nutrients tend to accumulate at interfaces between water and solid surfaces or at the air-water interface. Recently, a study was conducted to examine the ability of *L. biflexa patoc* I to scavenge [$1-^{14}C$] stearic acid from the surface of a glass cylinder immersed in buffer solution (114). The investigators found that the leptospires removed the fatty acid from the glass surface with greater efficiency than other bacterial strains that were examined.

This efficiency may be related to the ability of *Leptospira* cells to adhere readily and reversibly to solid surfaces (48), thus enabling leptospires to colonize new surfaces when nutrients become depleted. The high surface-to-volume ratio characteristic of leptospire cells may also facilitate efficient nutrient uptake (134). It should be noted that long-chain fatty acids, the principal growth substrates of leptospires, are compounds that, by virtue of their chemical structure, tend to accumulate at interfaces.

The possibility that leptospires degrade cellular macromolecules as a strategy to prolong survival has not been explored extensively. *Leptospira* cells and cells of other spirochetes contain a high percentage of lipids as compared to other bacteria (104, 128). Since leptospires possess the enzymatic apparatus required for ATP-yielding catabolism of long-chain fatty acids, it is possible that these spirochetes generate maintenance energy by oxidizing endogenous lipids during periods of carbon and energy source starvation. This may also be true of pathogenic leptospires, because these spirochetes survive for con-

siderable periods of time in oligotrophic bodies of water after being shed by hosts (2).

A number of differences in the metabolic capabilities of saprophytic and pathogenic leptospires have been reported. For example, representative pathogenic serovars of *Leptospira* exhibit strong urease activity and utilize, as a sole nitrogen source, urea present in media at concentrations as high as 2%. The one saprophytic strain tested did not exhibit urease activity (109). It was suggested that the ability to thrive at high concentrations of urea may be one of the factors that allows pathogenic leptospires to persist in kidneys during leptospirosis (109).

Free-living and pathogenic strains of leptospires also produce different types of peroxidative enzymes. Many members of the biflexa (saprophytic) complex break down H_2O_2 (presumably generated during respiration) by means of a peroxidase enzymatic activity. These strains have only low levels of catalase. By contrast, *L. interrogans* (pathogenic) serovars generally exhibit high catalase activities and low peroxidase activity (6, 7, 45, 46). Members of the "biflexa" and "interrogans" complexes also differ in optimal growth temperatures (102), concentration of long-chain fatty acids and unsaturated fatty acids optimal for growth (106), sensitivity to the purine analog 8-azaguanine (103), lipase production (103), and aminopeptidase production (33, 141). Some or all of these differences may represent adaptations of the two leptospire complexes to different ecological circumstances.

HOST-ASSOCIATED SPIROCHETES

Spirochetes in the Rumen Ecosystem

The number of spirochetes present in bovine rumen fluid ranges between 0.4×10^8 and 2.0×10^8 cells per ml, as determined by an enumeration technique involving rifampin as a selective agent (176). Many of these spirochetes grow at the expense of plant polymers, which abound in the rumen ecosystem. Thus, bovine rumen fluid contains large numbers of spirochetes that ferment xylan, arabinogalactan, pectin, polygalacturonate, and starch (148). Furthermore, hydrolysis products of these plant polymers serve as growth substrates for many spirochetes present in the rumen.

For example, it was reported that D-galacturonate, D-xylose, L-arabinose, D-glucose, and D-galactose each supported growth of between 0.2×10^6 and 4×10^6 spirochetes present per ml of bovine rumen fluid (148). These are minimum values representing only those rumen fluid spirochetes capable of growth under the cultural conditions used. Also, the values do not include spirochetes adhering to rumen epithelial surfaces or to forage particles undergoing degradation (148). Cellulose-degrading spirochetes have not been found in rumen fluid. However, many spirochetes present in rumen fluid grow at the expense of cellobiose, a hydrolysis product of cellulose (148).

All rumen spirochetes that have been isolated are obligate anaerobes that utilize only carbohydrates as carbon and energy sources. None has been isolated that can use other compounds (e.g. amino acids) as fermentable substrates for growth (148). Because of the high numbers of polysaccharide-degrading spirochetes present in rumen fluid, it seems likely that these bacteria contribute significantly to the degradation of plant polymers ingested by ruminants.

Spirochetes differing in morphology and physiology inhabit the bovine rumen (148, 176, 209). Rumen spirochetes have a wide spectrum of cell sizes and large differences in the number of periplasmic fibrils per cell. Thus, in bovine rumen fluid there are small spirochetes (0.1–0.2 by 5–8 μm) with two periplasmic fibrils per cell, intermediate size spirochetes (0.3–0.4 by 5–8 μm) with two or eight periplasmic fibrils per cell, and larger spirochetes with diverse cell morphologies. The largest strains have cells that measure 0.7 by 12–20 μm and possess 32 or more periplasmic fibrils arranged in a bundle (148, 176).

Paster & Canale-Parola (148) studied the physiological characteristics of 13 strains of rumen spirochetes. The strains could be divided into two groups on the basis of the fermentation end products formed from glucose. One group (six strains) fermented glucose mainly to formate, acetate, and succinate, whereas the other group (seven strains) produced formate, acetate, and ethanol but did not form succinate. The group of ethanol-forming spirochetes was quite heterogeneous with respect to the G+C content of the DNA (41–54 mol%) of the individual strains, their substrate utilization patterns, and their cell morphologies. These studies clearly demonstrated that the bovine rumen is inhabited by a wide diversity of spirochetes. According to current classification criteria (28), some of these spirochetes represent previously undescribed species of *Treponema*.

Stanton & Canale-Parola (177) reported that *Treponema bryantii*, a succinate-producing rumen spirochete, associated and interacted with cellulolytic bacteria from rumen fluid. The spirochete did not ferment cellulose but grew in coculture with a cellulolytic rumen bacterium in media that contained cellulose as the carbon and energy source (177). In these media, *T. bryantii* utilized, as fermentable substrates, soluble carbohydrates released from cellulose by the cellulolytic bacteria. The spirochete enhanced cellulose breakdown by the rumen cellulolytic isolate when the two organisms were cocultured in agar media.

Although the mechanism responsible for the enhancement of cellulose degradation is not clear at present, it has been suggested that in these cocultures the spirochetes are chemotactically attracted to areas of cellulose degradation. There, as a result of their incessant motion, they may randomly push the nonmotile cellulolytic bacteria toward undegraded cellulose fibers, thus enhancing cellulose breakdown (177). Electron microscopy observations have

indicated that spirochetes associate with the surface of cellulosic materials undergoing bacterial degradation in the rumen (53). Thus, it seems likely that interactions similar to those that occur in cocultures of *T. bryantii* and cellulolytic bacteria take place in the rumen. These interactions may affect the rate and extent of degradation of plant materials by cellulolytic bacteria in the rumen.

Pectinolytic rumen spirochetes release extracellular enzymes that function in the de-esterification and depolymerization of pectin (206, 209). These enzymes include pectin methylesterase (EC 3.1.1.11), pectate lyase (EC 4.2.2.2) and probably, in some cases, the hydrolytic enzyme polygalacturonase (EC 3.2.1.15). Some of the pectin-fermenting spirochetes utilize, as carbon and energy sources, a variety of carbohydrates, in addition to pectin (208, 209). Others appear to be much more limited nutritionally, because they ferment pectin but do not ferment any other substrate tested (209).

Nutritional Diversity of Human Oral Spirochetes

The gingival crevice–gingival sulcus region of humans, which comprises the narrow fissure between the gingiva and the tooth enamel, is inhabited by an enormous number of diverse bacteria. Investigators have counted, by light microscopy, an average of 1.3×10^{11} bacterial cells per gram (wet weight) of human gingival crevice debris (169). In clinically healthy sites of the crevice-sulcus regions, spirochetes are present in relatively small numbers (127).

However, in sites affected by periodontal disease[1], the numbers of spirochetes present in subgingival regions are much greater (127). The increase in number of spirochetes present in subgingival regions during periodontal disease has been clearly demonstrated by Listgarten & Helldén (127). These researchers collected samples of subgingival bacteria from normal and from periodontally diseased sites of each of 12 human subjects. The samples were examined by dark-field microscopy, and bacteria were counted microscopically and classified into nine morphological categories.

Listgarten & Helldén found spirochetes in the healthy sites of only 4 of the 12 subjects studied. In samples from healthy sites, spirochetes constituted an average of 1.8% of all bacterial cells present. In contrast, spirochetes were detected in all samples from periodontal pockets, in proportions ranging from 24.5% to 58% of the subgingival microbiota (mean value: 37.7%). Although there is ample evidence implicating microorganisms as primary etiologic agents of human periodontal disease, it is not clear whether specific microorganisms are responsible for the disease.

Thus, it is not known whether spirochetes play an important role in the initiation and progression of periodontal disease, or whether they are secondary

[1]Periodontal disease is a "chronic inflammation in the tooth supporting tissues that causes tissue destruction and eventuates in tooth loss" (129).

invaders that increase in number in response to environmental changes resulting from the development of periodontal disease. In any case, there seems to be no doubt that periodontal pockets are favorable ecological niches for spirochetes.

Because of the extremely high density of the microbial population present in the gingival crevice region, severe competition for growth substrates must exist among microorganisms that reside in that environment. It may be inferred that bacteria indigenous to the gingival crevice have developed special physiological adaptations that allow them to survive in spite of extensive fluctuations in the levels of available nutrients. A type of adaptation that may provide a significant competitive advantage is that found in *Treponema denticola*, an anaerobe commonly present in the gingival crevice.

This spirochete possesses an abundance of catabolic pathways that enable it to generate ATP by metabolizing a relatively wide spectrum of substrates. *T. denticola* catabolizes a variety of amino acids, including L-cysteine, glycine, L-serine, L-alanine, L-arginine, L-citrulline, L-histidine, and L-glutamate (19, 34, 93, 124). In addition, *T. denticola* ferments carbohydrates (e.g. glucose) via the Embden-Meyerhof pathway (93).

In contrast to the metabolic versatility of *T. denticola*, certain other oral spirochetes are quite limited with respect to the number of growth substrates they utilize. There are, for example, oral pectinolytic spirochetes that ferment, in addition to pectin, only the related compound polygalacturonate and the uronic acid D-glucuronic (168, 201). Some of the pectinolytic strains also ferment D-galacturonate, a hydrolysis product of pectin. The pectinolytic spirochetes do not utilize, as fermentable substrates, amino acids, neutral sugars, and all other compounds tested (201). Even though these spirochetes are quite specialized with respect to the fermentable substrates they use, they compete successfully with other microorganisms in their habitat, as indicated by the fact that they are commonly present in subgingival plaque.

Weber & Canale-Parola (202) suggested that pectinolytic spirochetes may grow at the expense of products formed through the metabolic activities of other bacteria present in the subgingival environment. For example, some of the bacteria that reside in the gingival crevice region produce extracellular enzymes that hydrolyze glucuronic acid–containing polymers (e.g. hyaluronic acid, chondroitin sulfate), which are constituents of human tissue (158, 165). It is possible that glucuronic acid and other compounds released by the activities of such enzymes serve as fermentable substrates for pectinolytic spirochetes in the subgingival regions.

This suggestion is supported by the observation that, even though oral pectinolytic spirochetes do not ferment hyaluronic acid, they grow in coculture with a hyaluronidase-producing strain of *Peptostreptococcus* in a medium containing hyaluronic acid as the fermentable substrate (202). This type of

microbial interaction may enhance the breakdown of tissue polymers and, therefore, may increase the rate of tissue destruction during periodontal disease (202). In addition, these interactions may also account for the large number of spirochetes present in periodontal pockets (202).

Oral pectinolytic spirochetes produce extracellular enzymes that catalyze reactions resulting in the depolymerization of pectin. One of these enzymes is pectin methylesterase (EC 3.1.1.11), which functions in removing methyl ester groups from pectin, and another is a pectate lyase (EC 4.2.2.2) of the endo type, which breaks down the de-esterified polymer, forming unsaturated products (201, 202). In addition, cell-associated lyase activity that catalyzes polygalacturonate breakdown was detected in pectinolytic oral spirochetes (202).

It is not known with certainty what compounds are used as fermentable substrates by oral spirochetes other than *T. denticola* and the pectinolytic strains. Although there is evidence suggesting that oral spirochetes such as *T. vincentii* and *T. scoliodontum* ferment amino acids and do not ferment carbohydrates (167), this has not been rigorously established.

T. denticola and *T. vincentii* have been surveyed for their ability to produce enzymes that are potentially destructive to host tissue (119). Both spirochetes produced C-8 esterase, acid phosphatase, and phosphoamidase activities. Furthermore, *T. denticola* strains produced a trypsin-like activity (119) and an extracellular fibrinolytic enzyme activity (145). Proteolytic activity probably increases the level of amino acids available to *T. denticola* as growth substrates.

Antigenic Variation in Borrelia

Relapsing fever is characterized by episodes of high fever and headaches, which persist for 3–7 days and then subside and recur after an interval of several days to several weeks (30). An initial attack may be followed by as many as ten relapses, especially in patients that are not treated (30). During the febrile phases of the illness, *Borrelia* are present in the blood and may be seen in wet mount preparations of the patient's blood examined by light microscopy. The spirochetes disappear from the blood stream during periods of remission. The ability of borreliae to circumvent immune surveillance and trigger repeated attacks in their animal hosts is due to a capacity of these organisms to undergo antigenic variation.

Early investigators observed that a qualitative change in the serological responses of animals occurred as each successive peak spirochete population appeared during consecutive relapses of relapsing fever (44, 56, 164). These observations suggested that the surface antigens of the organisms shifted and growth of a new population with alternative antigens was responsible for each relapse. Despite these tantalizing early results, few intensive investigations of

the relapse phenomenon have been carried out. Recently, with the development of media for cultivating *Borrelia* (115), as well as the development of procedures for isolating and identifying distinct *Borrelia* serotypes (183), investigators have begun to characterize the serological manifestations of relapsing fever in detail.

Stoenner and coworkers (183) have subcloned *B. hermsii* from successive relapse populations of spirochetes occurring in a single mouse. The subcloned spirochetes were used as immunogens for preparation of fluoresceinated antisera. A battery of 24 specific antisera prepared from relapse populations originating from a single *Borrelia* cell were then used to determine the order and frequency with which antigenic conversions occurred during infection. The investigators concluded that the dynamics of the relapse phenomenon are extremely complex. For example, during a relapse event, a *Borrelia* population consisting of a single serotype can change suddenly to a mixture of as many as seven serotypes. Generally, a given population of serotypes persisted for two or three days before being replaced by a new population of serotypes.

The investigators found a wide variation in the frequency of occurrence of different antigenic types. However, they determined statistically that some serotypes predominated in frequency over other serotypes during conversion, a finding that suggested that conversions did not occur entirely at random. Other experiments showed that *Borrelia* cultivated in vitro from a single cell inoculum spontaneously converted to yield stationary phase cultures containing cells of a mixture of serotypes. This suggested that the conversion phenomenon is independent of antibody production by the infected host. The rate of serotypic conversion during in vivo and in vitro cultivation was estimated to be about 1 in 10^3 to 10^4 per cell per generation.

In order to characterize antigenic variation in more detail, Barbour et al (10) analyzed the major variable proteins of four *B. hermsii* serotypes. Fractionation of whole cell lysates by SDS-polyacrylamide gel electrophoresis revealed the presence of a single major protein species (designated pI) unique to each serotype. By using western blot analysis, the investigators found that monoclonal antibodies specific for a given *Borrelia* serotype reacted only with the homologous pI protein. These studies showed that the pI proteins were serotype-specific antigens. A detailed analysis and comparison of the structures and amino acid sequences of these antigens should provide clues about the mechanism of antigenic variation in *B. hermsii*. Perhaps more importantly, the demonstration that serotype-specific antigens can be identified by western blot analysis provides a procedure to screen cloned pieces of *Borrelia* DNA for segments of genetic information specifying the conversion process.

While work on antigenic variation in *Borrelia* is still in its initial phases, it is now technically feasible to analyze the mechanism of conversion at the molecular level. This type of analysis will be useful in conducting interesting compara-

tive studies on mechanisms of antigenic variation in *Borrelia*, the African trypanosomes, *Neisseria gonorrhoeae*, *Salmonella* species, and other organisms (20). Most likely some aspects of the mechanism of antigenic variation in *Borrelia* represent specific adaptations that confer survival advantages to these arthropod-borne spirochetes in the natural environments where they exist.

At this writing there is no published evidence indicating that spirochetes borne by ixoides ticks (e.g. Lyme disease spirochetes) exhibit antigenic variation.

Motile Behavior and Attachment

Spirochetes retain vigorous motility in environments of sufficient viscosity to immobilize flagellated bacteria (71, 72, 111, 151). The coiled shape of spirochete cells plays an important role in the ability of these bacteria to move through viscous environments. This was shown by Greenberg & Canale-Parola (71), who found that motile mutant spirochetes that had little or no cell coiling could not swim in viscous environments through which the coiled parental strain moved readily. Furthermore, tightly coiled spirochete strains were able to swim through viscous solutions that immobilized loosely coiled strains.

Bacteria such as spiroplasmas, which have coiled cells but lack periplasmic fibrils and other obvious motility organelles, exhibit translational motility in environments of elevated viscosity, but not in liquids of viscosity comparable to that of water (204). The coiled shape of cells may be more important for motility in viscous liquids than the presence of endocellular motility organelles (e.g. periplasmic fibrils) that can function unimpeded by the viscosity of the external environment.

The ability to retain motility, and presumably chemotactic behavior, in viscous environments may confer competitive advantages to spirochetes. *Critispira* (see section on Distribution of Spirochetes in Nature) is an example of a spirochete that thrives in a viscous habitat. This large spirochete is the sole or principal inhabitant of the crystalline style of molluscs, a gelatinous, rod-shaped structure composed of mucoprotein secretions (22, 118, 189). It has been suggested that the viscosity of the style offers a selective environment that allows the proliferation of *Cristispira* and excludes competing bacteria (118). Spirochetes indigenous to other animals and to humans are also observed to swim freely through viscous environments (e.g. in gingival crevice fluids, mucosal fluids, rumen contents, and the gut contents of termites).

Some spirochetes present in the intestine of termites have a propulsive function for protozoa. These spirochetes are attached by one of their cell ends to the body surface of protozoa and perform an undulatory motion that propels the protozoan cells. This type of ectosymbiotic association has been reviewed extensively (22, 35). Canale-Parola (35) suggested that the attached spirochetes may not only provide locomotion for the protozoa but may also play a

role in the tactic responses and in the nutrition of the protozoan-spirochete consortium.

Other close physical associations of spirochetes with eukaryotes are those that occur with epithelial cells of the large intestine of various animals (79 and references therein). Electron microscope studies have revealed the presence of spirochetes in the brush border region of the epithelium that lines the colon of monkeys (142, 187, 188). The spirochetes were oriented perpendicularly to the mucosal surface so that one end of each spirochete deeply indented the surface of individual columnar cells lining the mucosa. The spirochetes caused major structural alterations in the animal host tissue as a result of their attachment and in many cases appeared to almost totally displace host-cell microvilli.

Although the outer sheath of the spirochete cell was closely associated with the columnar cell membranes, bacterial and host membranes were always separated by a uniform 6- to 8-nm gap containing extracellular material (142). The epithelial cells of the large intestine seemed to function adequately under these circumstances, as the spirochete-colonized monkeys were healthy and free of diarrhea or other intestinal disorders (142, 188). Spirochetal infestations of the human colon closely resemble those described for monkeys (139, 186). Although spirochetes have been isolated from intestinal contents (112, 161, 193) and rectal biopsies of humans (95), it is not clear that these isolates represent the same organisms as those that were observed to occur in association with primate intestinal epithelia. Furthermore, cultivated intestinal spirochetes have not been extensively characterized in terms of metabolic requirements or growth characteristics.

Thus, only speculations are possible as to benefits spirochetes may derive by inhabiting the microenvironment of the brush border. One possibility is that, by associating with the enzyme-containing glycocalyx that covers the microvilli of the columnar cells of the large intestine, spirochetes may be able to preferentially utilize nutrients that are absorbed, concentrated, and degraded in this region before passing into host-cell tissue (142).

The association mentioned above, involving attachment of spirochetes to epithelial cells in the intestine of monkeys, is mutualistic and does not seem to entail any sort of pathogenic interactions. The causative agent of syphilis, *T. pallidum*, attaches specifically to host cells, and there is evidence to suggest that the ability to attach influences the pathogenicity of this spirochete (59, 61, 86). *T. pallidum* cells extracted from infected rabbit testes attach via one or both ends to the membrane surfaces of various kinds of cultured animal cells. The ability to attach was found to be a property of *T. pallidum* specifically, as 11 nonpathogenic strains of treponemes failed to attach to cultured cells (61).

In addition, immune syphilitic rabbit serum reduced attachment of *T. pallidum* to host cells (86). Investigators have recently begun to identify treponemal proteins that may mediate attachment of *T. pallidum* to host-cell receptors (1,

11, 12, 60, 150). Although there is no direct evidence that attachment is a virulence factor for *T. pallidum*, attachment to various cell lines prolongs spirochete survival, and adherence may permit colonization of *T. pallidum* on selective sites of target tissue (61, 62, 160). It has been suggested that adherence may also allow *T. pallidum* to escape immune surveillance (13).

Investigators have recently cloned segments of *T. pallidum* DNA in *E. coli*, and analysis of antigens encoded by *T. pallidum* genes is underway (173–175, 197, 200). The availability of large amounts of treponemal antigens made possible by recombinant DNA technology circumvents problems associated with the inability to culture *T. pallidum* in vitro to high yields. By using cloned *T. pallidum* genes, it should be possible to study in detail some of the proteins that mediate attachment to host-cell surfaces.

Bacteriophages and Plasmids

Genetic analysis has played a crucial role in elucidating physiological mechanisms that have a bearing on the ecology of bacteria. However, to date no system for genetic exchange has been developed for any spirochete species. Numerous reports of bacteriophage-like particles associated with different species of spirochetes suggest that the potential for genetic exchange exists for at least some strains of these bacteria. Ritchie and coworkers (157) surveyed a collection of *T. hyodysenteriae* strains by electron microscopy and found particles with morphologies typical of bacteriophages. They identified a common phage type having a round head and simple tail in 18 spirochete cultures. Phage-like entities have also been reported to be associated with *L. illini* (156), the *Ixodes dammini* spirochete (85), and with unidentified species of *Treponema* from pigs (17, 159).

Masuda & Kawata (133) detected large hexagonal particles in cultures of *T. phagedenis* (the Reiter treponeme) after treatment with mitomycin C, an observation that suggests that the organisms may have contained a lysogenic bacteriophage. However, these investigators were unable to propagate the bacteriophage-like particles they observed. While to date no one has reported the propagation of a spirochete phage, it is likely that isolation and assay procedures leading to the recovery of spirochete phages will be developed in the future. Availability of suitable bacteriophages may provide investigators with a system of genetic exchange by transduction in spirochetes.

The potential role of plasmids as in vivo genetic exchange mechanisms in spirochetes seems less clear. It has been reported that *T. pallidum* contains plasmid DNA (146). Apparently, plasmids are not present in cells of the free-living spirochetes *S. aurantia* and *S. halophila* (B. J. Paster, personal communication). Inasmuch as only a few spirochetes have been examined for the presence of extrachromosomal DNA, conclusions cannot be drawn with regard to the distribution of plasmids among spirochetes.

CONCLUDING REMARKS

In this review we have discussed numerous studies that dealt with aspects of the ecology of spirochetes. It is apparent, however, that many questions relating to the ecology of these bacteria remain unresolved, and a great many others have not yet been addressed. One example is the problem of identifying factors that are responsible for the pathogenicity of virulent spirochetes. Possible virulence factors, such as antigenic variation and attachment to host cells, have been identified in some spirochetes. However, studies of the mechanisms underlying these physiological adaptations are still in their initial stages and, at this writing, almost nothing is known about factors that contribute to the virulence of other pathogenic spirochetes, including *T. hyodysenteriae*, the leptospires, and the Lyme disease spirochete.

Another aspect of the ecology of spirochetes that has not been addressed is the question of how much spirochetes contribute, as compared to other microorganisms, to the overall breakdown and transformation of organic material (e.g. plant polysaccharides, tissue polymers, and other compounds) in natural environments. Quantitative answers to this question would not only increase our understanding of the dynamics of microbial interactions in selected environments, but, in the case of host-associated spirochetes, could help define factors that influence the nutrition of the host animal.

Although our present knowledge of the ecology of spirochetes is very limited, during the last 15 years significant progress has been made in understanding the relationships of spirochetes to their environments. For example, we have gained an appreciation for the roles played by spirochetes in the biochemical transformations that take place in various aerobic and anaerobic habitats, including freshwater and marine habitats, as well as habitats within the bodies of animals and humans. In addition, we have learned about physiological processes expressed by spirochetes under conditions that occur commonly in nature, such as conditions of starvation and of limited availability of nutrients.

Much of the work on the ecology of spirochetes has been carried out in the laboratory, very little in the field. Unquestionably there is need for both of these experimental approaches in studying the ecology of any group of microorganisms. As pointed out by Brock (25), " . . . field and laboratory can complement each other. The microbial ecologist can have his roots in the soil, but his feet in the laboratory." Appropriate methodology needs to be developed so that field studies on the ecology of spirochetes can be carried out. On the other hand, with the advent of recombinant DNA technology, new methods for selectively culturing and enumerating spirochetes, advanced immunological techniques, and other improved experimental approaches, the tools are now available to answer many questions relating to the ecology of spirochetes.

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Literature Cited

1. Alderete, J. F., Baseman, J. B. 1980. *Infect. Immun.* 30:814–23
2. Alexander, A. D. 1980. See Ref. 123, pp. 376–82
3. Allen, S. L., Johnson, R. C., Petersen, D. 1971. *Infect. Immun.* 3:727–34
4. Antonakopoulos, G., Newman, J., Wilkinson, M. 1982. *Histopathology* 6:477–88
5. Armitage, G. C., Dickinson, W. R., Jenderseck, R. S., Levine, S. M., Chambers, D. W. 1982. *J. Periodont.* 53:550–56
6. Austin, F. E., Barbieri, J. T., Corin, R. E., Grigas, K. E., Cox, C. D. 1981. *Infect. Immun.* 33:372–79
7. Banfi, E., Cinco, M., Dri, P. 1981. *Experientia* 37:147–48
8. Barbieri, J. T., Cox, C. D. 1981. *Infect. Immun.* 31:992–97
9. Barbour, A. G., Burgdorfer, W., Hayes, S. F., Péter, O., Aeschlimann, A. 1983. *Current Microbiol.* 8:123–26
10. Barbour, A. G., Tessier, S. L., Stoenner, H. G. 1982. *J. Exp. Med.* 156:1312–24
11. Baseman, J. B., Alderete, J. F. 1983. See Ref. 163, pp. 229–39
12. Baseman, J. B., Hayes, E. C. 1980. *J. Exp. Med.* 151:573–86
13. Baseman, J. B., Hayes, N. S., Spitznagel, J. K., Hayes, E. C. 1979. In *Microbiology—1979*, ed. D. Schlessinger, pp. 203–8. Washington DC: Am. Soc. Microbiol.
14. Baseman, J. B., Nichols, J. C., Hayes, N. S. 1976. *Infect. Immun.* 13:704–11
15. Benach, J. L., Bosler, E. M., Hanrahan, J. P., Coleman, J. L., Habicht, G. S., et al. 1983. *N. Engl. J. Med.* 308:740–42
16. Berg, H. C., Turner, L. 1979. *Nature* 278:349–51
17. Berthiaume, L., Elazhary, Y., Alain, R., Ackerman, H.-W. 1979. *Can. J. Microbiol.* 25:114–16
18. Blakemore, R. P., Canale-Parola, E. 1973. *Arch. Mikrobiol.* 89:273–89
19. Blakemore, R. P., Canale-Parola, E. 1976. *J. Bacteriol.* 128:616–22
20. Bloom, B. R. 1979. *Nature* 279:21–26
21. Bosler, E. M., Coleman, J. L., Benach, J. L., Massey, D. A., Hanrahan, J. P., et al. 1983. *Science* 220:321–22
22. Breznak, J. A. 1973. *CRC Critical Rev. Microbiol.* 2:457–89
23. Breznak, J. A., Canale-Parola, E. 1969. *J. Bacteriol.* 97:386–95
24. Breznak, J. A., Canale-Parola, E. 1975. *Arch. Microbiol.* 105:1–12
25. Brock, T. D. 1966. *Principles of Microbial Ecology*. Englewood Cliffs, NJ: Prentice-Hall. 306 pp.
26. Bromley, D. B., Charon, N. W. 1979. *J. Bacteriol.* 137:1406–12
27. Bryant, M. P., Wolin, M. J. 1975. In *Proceedings of the First Intersectional Congress of the International Association of Microbiology Societies*. 2:297–306. Tokyo: Developmental, Microbiology, Ecology, Science Council of Japan.
28. Buchanan, R. E., Gibbons, N. E., eds. 1974. *Bergey's Manual of Determinative Bacteriology*. Baltimore: Williams & Wilkins, 8th ed.
29. Burgdorfer, W. 1976. See Ref. 100, pp. 191–200
30. Burgdorfer, W. 1980. See Ref. 123, pp. 383–88
31. Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., et al. 1982. *Science* 216:1317–19
32. Burgdorfer, W., Barbour, A. G., Hayes, S. F., Péter, O., Aeschlimann, A. 1983. *Acta Tropica* 40:79–83
33. Burton, G., Blenden, D. C., Goldberg, H. S. 1970. *Appl. Microbiol.* 19:586–88
34. Canale-Parola, E. 1977. *Bacteriol. Rev.* 41:181–204
35. Canale-Parola, E. 1978. *Ann. Rev. Microbiol.* 32:69–99
36. Canale-Parole, E. 1981. See Ref. 178, pp. 538–47
37. Canale-Parola, E., Holt, S. C., Udris, Z. 1967. *Arch. Mikrobiol.* 59:41–48
38. Canale-Parola, E., Kidder, G. W. 1982. *J. Bacteriol.* 152:1105–10
39. Canale-Parola, E., Udris, Z., Mandel, M. 1968. *Arch. Mikrobiol.* 63:385–97
40. Cinco, M., Ivanov, I. 1978. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. A* 240:356–58
41. Cinco, M., Sottocasa, G. L. 1976. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. A* 236:354–58
42. Cinco, M., Tamaro, M., Cociancich, L. 1975. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. A* 233:400–5
43. Cinco, M., Tamaro, M., Rottini, G. D., Monti-Bragadin, C. 1974. *Int. J. Syst. Bacteriol.* 24:131–35
44. Coffey, E. M., Eveland, W. C. 1967. *J. Infect. Dis.* 117:29–34
45. Corin, R. E., Boggs, E., Cox, C. D. 1978. *Infect. Immun.* 22:672–75
46. Corin, R. E., Cox, C. D. 1980. *Can. J. Microbiol.* 26:121–29
47. Cox, C. D. 1983. See Ref. 163, pp. 57–70
48. Cox, P. J., Twigg, G. I. 1974. *Nature* 250:260–61
49. Cwyk, W. M., Canale-Parola, E. 1979. *Arch. Microbiol.* 122:231–39
50. Dawes, E. A. 1976. *Symp. Soc. Gen. Microbiol.* 26:19–53
51. Deinema, M. 1976. *Can. J. Microbiol.* 22:1768–71
52. Dikken, H., Kmety, E. 1978. In *Methods in Microbiology*, ed. T. Bergan, J. R. Norris, 11:259–307. London: Academic
53. Dinsdale, D., Morris, E. J., Bacon, J. S. D. 1978. *Appl. Environ. Microbiol.* 36:160–68
54. Faine, S., Stallman, N. D. 1982. *Int. J. Syst. Bacteriol.* 32:461–63
55. Felsenfeld, O. 1965. *Bacteriol. Rev.* 29:46–74
56. Felsenfeld, O., Decker, W. J., Wohlhieter, J. A., Rafyi, A. 1965. *J. Immunol.* 94:805–17
57. Fieldsteel, A. H., Cox, D. L., Moeckli, R. A. 1982. *Infect. Immun.* 35:449–55
58. Fitzgerald, T. 1981. *Bull. WHO* 59:787–812
59. Fitzgerald, T. J. 1981. *Ann. Rev. Microbiol.* 35:29–54
60. Fitzgerald, T. J. 1983. See Ref. 163, pp. 195–227
61. Fitzgerald, T. J., Johnson, R. C., Miller, J. N., Sykes, J. A. 1977. *Infect. Immun.* 18:467–78
62. Fitzgerald, T. J., Miller, J. N., Sykes, J. A. 1975. *Infect. Immun.* 11:1133–40
63. Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., et al. 1980. *Science* 209:457–63
64. Gaber, M. S., Khalil, G. M., Hoogstraal, H. 1982. *Exp. Parasitol.* 54:182–84
65. Gad, A., Willén, R., Furugård, K., Fors, B., Hradsky, M. 1977. *Upsala J. Med. Sci.* 82:49–54
66. George, H. A., Smibert, R. M. 1982. *J. Bacteriol.* 152:1049–59
67. George, H. A., Smibert, R. M. 1982. *J. Bacteriol.* 152:1060–65
68. Glock, R. D., Harris, D. L. 1972. *Vet. Med. Small Anim. Clin.* 67:65–68
69. Greenberg, E. P., Canale-Parola, E. 1976. *Arch. Microbiol.* 110:185–94
70. Greenberg, E. P., Canale-Parola, E. 1977. *J. Bacteriol.* 130:485–94
71. Greenberg, E. P., Canale-Parola, E. 1977. *J. Bacteriol.* 131:960–69
72. Greenberg, E. P., Canale-Parola, E. 1977. *J. Bacteriol.* 132:356–58
73. Gronlund, A. F., Campbell, J. J. R. 1965. *J. Bacteriol.* 90:1–7
74. Haapala, D. K., Rogul, M., Evans, L. B., Alexander, A. D. 1969. *J. Bacteriol.* 98:421–28
75. Hanson, L. E. 1982. *J. Am. Vet. Med. Assoc.* 181:1505–9
76. Hanson, L. E., Tripathy, D. N., Evans, L. B., Alexander, A. D. 1974. *Int. J. Syst. Bacteriol.* 24:355–57
77. Harder, W., Dijkhuizen, L. 1983. *Ann. Rev. Microbiol.* 37:1–23
78. Harris, D. L., Glock, R. D., Christensen, C. R., Kinyon, J. M. 1972. *Vet. Med. Small Anim. Clin.* 67:61–64
79. Harris, D. L., Kinyon, J. M. 1974. *Am. J. Clin. Nutrition* 27:1297–304
80. Harwood, C. S., Canale-Parola, E. 1981. *J. Bacteriol.* 148:109–16
81. Harwood, C. S., Canale-Parola, E. 1981. *J. Bacteriol.* 148:117–23
82. Harwood, C. S., Canale-Parola, E. 1982. *J. Bacteriol.* 152:246–54
83. Harwood, C. S., Canale-Parola, E. 1983. *Int. J. Syst. Bacteriol.* 33:573–79
84. Harwood, C. S., Jannasch, H. W., Canale-Parola, E. 1982. *Appl. Environ. Microbiol.* 44:234–37
85. Hayes, S. F., Burgdorfer, W., Barbour, A. G. 1983. *J. Bacteriol.* 154:1436–39
86. Hayes, N. S., Muse, K. E., Collier, A. M., Baseman, J. B. 1977. *Infect. Immun.* 17:174–86
87. Hazelbauer, G. L., Harayama, S. 1983. *Int. Rev. Cytol.* 81:33–70
88. Hellstrom, J. S., Marshall, R. B. 1978. *Res. Vet. Sci.* 25:29–33
89. Henneberry, R. C., Baseman, J. B., Cox, C. D. 1970. *Antonie van Leeuwenhoek* 4:489–501
90. Henneberry, R. C., Cox, C. D. 1970. *Can. J. Microbiol. 16*:41–45
91. Henry, R. A., Johnson, R. C. 1978. *Appl. Environ. Microbiol.* 35:492–99
92. Hespell, R. B., Canale-Parola, E. 1970. *Arch. Mikrobiol.* 74:1–18
93. Hespell, R. B., Canale-Parola, E. 1971. *Arch. Mikrobiol.* 78:234–51

94. Holt, S. C. 1978. *Microbiol. Rev.* 42:114–60
95. Hovind-Hougen, K., Birch-Anderson, A., Henrik-Nielsen, R., Orholm, M., Pedersen, J. O., et al. 1982. *J. Clin. Microbiol.* 16:1127–36
96. Hufnagel, L. A., Myhal, M. L. 1977. *Trans. Am. Micros. Soc.* 96:406–11
97. Jacob, E., Meiller, T. F., Naumann, R. K. 1982. *J. Periodont. Res.* 17:145–53
98. Jenkin, H. M., Sandok, P. L. 1983. See Ref. 163, pp. 71–98
99. Joens, L. A., Kinyon, J. M. 1982. *J. Clin. Microbiol.* 15:994–97
100. Johnson. R. C., ed. 1976. *The Biology of Parasitic Spirochetes*. New York: Academic. 402 pp.
101. Johnson, R. C. 1981. See Ref. 178, pp. 582–91
102. Johnson, R. C. Harris, V. G. 1967. *J. Bacteriol.* 94:27–31
103. Johnson, R. C., Harris, V. G. 1968. *Appl. Microbiol.* 16:1584–90
104. Johnson, R. C., Livermore, B. P., Walby, J. K., Jenkin, H. M. 1970. *Infect. Immun.* 2:286–91
105. Johnson, R. C., Rogers, P. 1964. *J. Bacteriol.* 87:422–26
106. Johnson, R. C., Walby, J. K. 1972. *Appl. Microbiol.* 23:1027–28
107. Joseph, R., Canale-Parola, E. 1972. *Arch. Mikrobiol.* 81:146–68
108. Joseph, R., Holt, S. C., Canale-Parola, E. 1973. *J. Bacteriol.* 115:426–35
109. Kadis, S., Pugh, W. L. 1974. *Infect. Immun.* 10:793–801
110. Kahan, A., Dougados, M., Vannier, A., Amor, B. 1983. *The Lancet* 2:174
111. Kaiser, G. E., Doetsch, R. N. 1975. *Nature* 255:656–57
112. Kaplan, C. R., Takeuchi, A. 1979. *J. Am. Med. Assoc.* 240:52–53
113. Kathariou, S., Greenberg, E. P. 1983. *J. Bacteriol.* 156:95–100
114. Kefford, B., Kjelleberg, S., Marshall, K. C. 1982. *Arch. Microbiol.* 133:257–60
115. Kelly, R. T. 1972. *Science* 173:443–44
116. Kelly, R. T. 1981. See Ref. 178, pp. 578–81
117. Kinyon, J. M., Harris, D. L. 1979. *Int. J. Syst. Bacteriol.* 29:102–9
118. Kuhn, D. A. 1981. See Ref. 178, pp. 555–63
119. Laughon, B. E., Syed, S. A., Loesche, W. J. 1982. *J. Clin. Microbiol.* 15:97–102
120. Lawry, E. V., Howard, H. M., Baross, J. A., Morita, R. Y. 1981. *Current Microbiol.* 6:355–60
121. Leach, W. D., Lee. A., Stubbs, R. P. 1973. *Infect. Immun.* 7:961–72
122. Lee, A., Phillips, M. 1978. *Appl. Environ. Microbiol.* 35:610–13
123. Lennette, E. H., ed. 1980. *Manual of Clinical Microbiology*. Washington DC: Am. Soc. Microbiol. 3rd ed.
124. Leschine, S. B., Canale-Parola, E. 1980. *Current Microbiol.* 3:305–10
125. Leschine, S. B., Canale-Parola, E. 1980. *Abstrs. Ann. Meet. Am. Soc. Microbiol.* I20
126. Leschine, S. B., Canale-Parola, E. 1980. *J. Clin. Microbiol.* 12:792–95
127. Listgarten, M. A., Helldén, L. 1978. *J. Clin. Periodontol.* 5:115–32
128. Livermore, B. P., Johnson., R. C. 1974. *J. Bacteriol.* 120:1268–73
129. Loesche, W. J. 1976. See Ref. 100, pp. 261–75
130. Lysko, P. G., Cox, C. D. 1977. *Infect. Immun.* 16:885–90
131. Macnab, R. M. 1982. *Symp. Soc. Exp. Biol.* 35:77–104
132. Margulis, L., To, L. P., Chase, D. G. 1981. See Ref. 178, pp. 548–54
133. Masuda, K., Kawata, T. 1979. *FEMS Microbiol. Letts.* 6:29–31
134. Matin, A. 1979. In *Strategies of Microbial Life in Extreme Environments*, ed. M. Shilo, pp. 323–39. New York: Verlag Chemie
135. Matthews, H. M., Yang, T.-K., Jenkin, H. M. 1980. *J. Bacteriol.* 143:1151–55
136. Miao, R., Fieldsteel, A. H. 1978. *J. Bacteriol.* 133:101–7
137. Miao, R. M., Fieldsteel, A. H. 1980. *J. Bacteriol.* 141:427–29
138. Miao, R. M., Fieldsteel, A. H., Harris, D. L. 1978. *Infect. Immun.* 22:736–39
139. Minio, F., Tonietti, G., Torsoli, A. 1973. *Rendic. Gastroenterol.* 5:183–95
140. Miura, H., Horiguchi, M., Matsumoto, T. 1980. *Appl. Environ. Microbiol.* 40: 294–300
141. Neill, S. D., Reid, L. R., Ellis, W. A. 1983. *J. Gen. Microbiol.* 129:395–400
142. Neutra, M. R. 1980. *J. Ultrastruct. Res.* 70:186–203
143. Nichols, J. C., Baseman, J. B. 1978. *Infect. Immun.* 19:854–60
144. Nielsen, R. H., Orholm, M., Pedersen, J. O., Hovind-Hougen, K., Teglbjaerg, P. S., Thaysen, E. H. 1983. *Gastroenterology* 85:62–67
145. Nitzan, D., Sperry, J. F., Wilkins, T. D. 1978. *Arch. Oral Biol.* 23:465–70
146. Norgard, M. V., Miller, J. N. 1981. *Science* 213:553–55
147. Paster, B. J., Canale-Parola, E. 1980. *J. Bacteriol.* 141:359–64
148. Paster, B. J., Canale-Parola, E. 1982. *Appl. Environ. Microbiol.* 43:686–93
149. Paster, B. J., Hespell, R. B., Woese, C. R. 1983. *Absts. Ann. Meet. Am. Soc. Microbiol.* I79
150. Peterson, K. M., Baseman, J. B., Alderete, J. F. 1983. *J. Exp. Med.* 157:1958–70
151. Petrino, M., Doetsch, R. N. 1978. *J. Gen. Microbiol.* 109:113–17
152. Picard, B., Lariviere, S., Saheb, S. A., 1980. *Can. J. Microbiol.* 26:985–91
153. Pirt, J. S. 1975. *Principles of Microbe and Cell Cultivation*. New York: Wiley, 274 pp.
154. Richards, C. S. 1978. *Trans. Am. Micros. Soc.* 97:191–98
155. Ris, D. R., Hamel, K. L. 1979. *New Zealand Med. J.* 90:212
156. Ritchie, A. E. 1976. See Ref. 100, pp. 19–37
157. Ritchie, A. E., Robinson, I. M., Joens, L. A., Kinyon, J. M. 1978. *Vet. Rec.* 102:34–35
158. Rosan, B., Williams, N. B. 1964. *Arch. Oral. Biol.* 9:291–98
159. Saheb, S. A. 1974. *Rev. Can. Biol.* 33:67–70
160. Sandok, P. L., Knight, S. T., Jenkin, H. M. 1976. *J. Clin. Microbiol.* 4:360–71
161. Sanna, A., Dettori, G., Grillo, R., Rossi, A., Chiarenza, D. 1982. *L'Igiene Moderna* 77:287–97
162. Savage, D. C., McAllister, J. S., Davis, C. P. 1971. *Infect. Immun.* 4:492–502
163. Schell, R. F., Musher, D. M., eds. 1983. *Pathogenesis and Immunology of Treponemal Infection*. New York: Dekker. 392 pp.
164. Schuhardt, V. T., Wilkerson, M. 1951. *J. Bacteriol.* 62:215–19
165. Schultz-Haudt, S. D., Scherp, H. W. 1956. *J. Dent. Res.* 35:299–307
166. Sirevåg, R., Ormerod, J. G. 1970. *Biochem. J.* 120:399–408
167. Smibert, R. M. 1981. See Ref. 178, pp. 564–77
168. Smibert, R. M., Burmeister, J. A. 1983. *Int. J. Syst. Bacteriol.* 33:852–56
169. Socransky, S. S., Gibbons, R. J., Dale, A. C., Bortnick, L., Rosenthal, E., Macdonald, J. B. 1963. *Arch. Oral Biol.* 8:275–80
170. Socransky, S. S., Listgarten, M., Hubersak, C., Cotmore, J., Clark, A. 1969. *J. Bacteriol.* 98:878–82
171. Songer, J. G., Harris, D. L. 1978. *Am. J. Vet. Res.* 39:913–16
172. Songer, J. G., Kinyon, J. M., Harris, D. L. 1976. *J. Clin. Microbiol.* 4:57–60
173. Stamm, L. V., Bassford, P. J. Jr. 1982. *DNA* 1:329–33
174. Stamm, L. V. Folds, J. D., Bassford, P. J. Jr. 1982. *Infect. Immun.* 36:1238–41
175. Stamm, L. V., Kerner, T. C. Jr., Bankaitis, V. A., Bassford, P. J. Jr. 1983. *Infect. Immun.* 41:709–21
176. Stanton, T. B., Canale-Parola, E. 1979. *Appl. Environ. Microbiol.* 38:965–73
177. Stanton, T. B., Canale-Parola, 1980. *Arch. Microbiol.* 127:145–56
178. Starr, M. P., Stolp, H., Truper, H. G., Balows, A., Schlegel, H. G. eds., 1981. *The Prokaryotes*, vol. I. New York: Springer. 1102 pp.
179. Steere, A. C., Grodzicki, R. L., Kornblatt, A. N., Craft, J. E., Barbour, A. G., et al. 1983. *N. Engl. J. Med.* 308:733–40
180. Steere, A. C., Malawista, S. E. 1979. *Ann. Intern. Med.* 91:730–33
181. Steere, A. C., Malawista, S. E., Hardin, J. A., Ruddy, S., Askenase, P. W., Andiman, W. A. 1977. *Ann. Intern. Med.* 86:685–98
182. Stoenner, H. G. 1974. *Appl. Microbiol.* 28:540–43
183. Stoenner, H. G., Dodd, T., Larsen, C. 1982. *J. Exp. Med.* 156:1297–311
184. Taber, L. H., Feigin, R. D. 1979. *Pediatric Clinics of North America.* 26:377–413
185. Taichman, N. S., Bohringer, H. R., Lai, C. H., Shenker, B. J., Tsai, C.-C., et al. 1982. *J. Periodont. Res.* 17:449–51
186. Takeuchi, A., Jervis, H. R., Nakazawa, H., Robinson, D. M. 1974. *Am. J. Clin. Nutrition* 27:1287–96
187. Takeuchi, A., Zeller, J. A. 1972. *J. Ultrastruct. Res.* 40:313–24
188. Takeuchi, A., Zeller, J. A. 1972. *Infect. Immun.* 6:1008–18
189. Tall, B. D., Naumann, R. K. 1981. *Appl. Environ. Microbiol.* 42:336–43
190. Tempest, D. W., Neijssel, O. M. 1981. In *Trends in the Biology of Fermentation for Fuels and Chemicals*, ed. A. Hollaender, pp. 335–56. New York: Plenum
191. Terracciano, J. S., Canale-Parola, E. 1983. *Abstrs. Ann. Meet. Am. Soc. Microbiol.* I69
192. To, L., Margulis, L., Cheung, A. T. W. 1978. *Microbios* 22:103–33
193. Tompkins, D. S., Waugh, M. W., Cooke, E. M. 1981. *J. Clin. Pathol.* 34:1385–87
194. Turek, J. J., Meyer, R. C. 1978. *Infect. Immun.* 20:853–55
195. Turek, J. J., Meyer, R. C. 1979. *Current Microbiol.* 3:27–31
196. Tyson, G. E. 1974. *Arch. Microbiol.* 99:281–94
197. vanEmbden, J. D., vanderDonk, H. J., vanEijk, R. V., vanderHeide, H. G., deJong, J. A., et al. 1983. *Infect. Immun.* 42:187–96
198. Veldkamp, H. 1960. *Antonie van Leeuwenhoek* 26:103–25
199. Veldkamp, H. 1977. In *Advances in*

Microbial Ecology, ed. M. Alexander, 1:59–94. New York: Plenum
200. Walfield, A. M., Hanff, P. A., Lovett, M. A. 1982. *Science* 216:522–23
201. Weber, F. H., Canale-Parola, E. 1983. *Abstrs. Ann. Meet. Am. Soc. Microbiol.* I113
202. Weber, F. H., Canale-Parola, E. 1984. *Appl. Environ. Microbiol.* In press
203. Weber, F. H., Greenberg, E. P. 1981. *Current Microbiol.* 5:303–6
204. Whitcomb, R. F. 1981. *Ann. Rev. Entomol.* 26:397–425
205. WHO Scientific Group. 1982. *Treponemal Infections*. WHO/Technical Report Series 674
206. Wojciechowicz, M., Ziolecki, A. 1979. *Appl. Environ. Microbiol.* 37:136–42
207. Zaher, M. A., Soliman, Z. R., Diab, F. M. 1977. *Z. Parasitenk.* 53:213–23
208. Ziolecki, A. 1979. *Appl. Environ. Microbiol.* 37:131–35
209. Ziolecki, A., Wojciechowicz, M. 1980. *Appl. Environ. Microbiol.* 39:919–22

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MOLECULAR CLONING OF BACTERIAL ANTIGENS AND VIRULENCE DETERMINANTS

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INTRODUCTION

Modern molecular genetics and biochemistry provide unique tools for the study of bacterial pathogenesis. The realization of gene transmission systems in bacteria and an understanding of extrachromosomal inheritance helped guide us toward the exploration of bacterial virulence using genetic methods. The finding that a significant number of virulence traits are plasmid-borne in bacteria allowed their ready genetic manipulation (34). The comparison of isogenic strains, with and without putative virulence plasmids, can be and has

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been highly instructive in understanding the genetic basis of colonization and pathogenicity (34, 74, 136, 143). However, pathogenicity is often determined by the net effect of several genes or groups of genes. Accordingly, the construction of a bacterial strain containing a toxin-encoding plasmid, followed by its comparison to a non–plasmid containing isogenic strain, would likely be useful but might not provide a total picture of the organism's pathogenic potential. Recombinant DNA methodology affords one the ability to systematically isolate those genes that may be contributing to pathogenicity. It enables one to begin a genetic analysis of a given pathogen irrespective of whether or not a traditional system for genetic exchange is available in that organism.

There are a number of reasons for applying recombinant DNA methodology to the study of bacterial pathogenicity. First, many virulence genes will not be plasmid-encoded but rather will reside on the host genome. Recombinant DNA techniques simplify our access to these genes. Sorting these genes out of genomic libraries established in *Esherichia coli* often is easily accomplished. Once the DNA fragment specifying the trait in question is identified, a great deal may be learned, regardless of whether it may or may not be reintroduced into the pathogenic host from which it was cloned. Deletion and insertion mutagenesis can be used to define the boundaries of the gene in question, assuming it is transcribed and translated in *E. coli*. Direct base sequence analysis (91, 123) may be applied to provide information on gene structure, including possible regulatory sequences. The amino acid sequence of the protein encoded by the gene may be deduced from the DNA base sequence. Usually this provides useful baseline information and the foundation for comparative studies. Cloned virulence genes provide the DNA probes that allow one to ask questions about the dissemination of such genetic information in organisms that are both closely and distantly related to the original host. These studies may yield clues relating to gene origin and gene flow in pathogens. Also, one may use cloned gene sequences as hybridization probes to ask questions about gene copy number and the presence of "silent" virulence genes.

Finally, cloned genes may be used as diagnostic probes in surveying, for example, potentially toxigenic strains in a clinical setting or elsewhere. When cloned gene sequences are transcribed and translated in the heterospecific host cell (usually *E. coli*), it should be possible to overproduce the specific antigen or virulence determinant in connection with immunogen preparation and vaccine development. Even though it is often possible to produce such immunogens from the pathogenic host, it may be safer to produce them using *E. coli*. A specific example is in the development of streptococcal vaccines, where such preparations may be contaminated with streptococcal antigens that cross-react with certain human antigens (60). Once a gene specifying an immunogen is cloned, it should be possible to modify that gene using site-specific mutagenesis techniques (129) to make it a molecule that is more nontoxic and more highly immunogenic. Another emerging area that centers on the possibility of cloning virulence determinants (specifically toxins) involves the fusion of cloned virulence genes to genes specifying proteins that recognize specific eucaryotic cell types. Such hybrid proteins may provide powerful specific toxin delivery systems for the treatment of certain diseases (e.g. neoplasms).

If one is able to reintroduce a cloned DNA fragment specifying an antigen or virulence determinant into the host of origin, then several new and important avenues of study are opened. The most obvious one is the possibility of evaluating the specific contribution of a determinant to pathogenicity. Assuming the availability of mutants that are deficient in the cloned trait, and an appropriate genetic transfer system, one can add the cloned gene back to the mutant and assess its effect on pathogenicity of the organism. This approach can be extended to include groups of genes by constructing appropriate recombinant plasmids in *E. coli* or by adding genes back into the host of origin one at a time, using plasmids that can coexist and are stable (i.e. are compatible) in the same cell. If mutants in presumed virulence or antigen genes are not available, then their cloned versions may help in obtaining them. When the information on the physical limits of the gene involved is available, a portion of the coding sequence can be interrupted (or substituted) with a genetic insertion, preferably one carrying a drug-resistance gene. This genetic construction can then be introduced into the wild-type host by an appropriate genetic transfer system. Its integration into the wild-type allele effects a disruption in the gene, rendering the host cell a mutant in the respective phenotype (34, 73, 120). Finally, if cloned genes can be reintroduced into the host of origin, the possibility of engineering bacterial strains that can serve as a source of effective and safer whole-cell vaccines becomes feasible. Indeed, such approaches might not involve the original host but some related organism to which a single property might be added for the desired result.

The above discussion adequately frames the perspective for employing molecular cloning strategies to study bacterial pathogens. It should be appreciated that most of the possibilities described already have been published in the scientific literature.

MOLECULAR CLONING METHODS APPLIED TO PATHOGENS

Recombinant DNA methodology has been reviewed in an excellent laboratory manual by Maniatis et al (90) and in *Methods in Enzymology*, edited by Wu (175). The newcomer to recombinant DNA methodology is referred to the introductory chapters of these books for a thorough introduction to molecular cloning theory and practice. Some recombinant DNA methodology that is especially relevant to cloning genes from pathogens is discussed in this section.

Generally, the cloning of genes specifying antigens or virulence determinants from bacteria will not provide the investigator with the ability to select directly in *E. coli* the desired DNA fragment.

It then becomes desirable to apply methods that will most efficiently develop a genomic library in the appropriate recombinant DNA host (usually *E. coli*). Cosmid gene cloning methods (90:295–307; 175:229–326) are popular and effective for this purpose. These methods enable relatively large chromosomal fragments to be captured using specialized cloning replicons, termed cosmids. Purified large genomic fragments, usually obtained by partial restriction endonuclease cleavage, are ligated into linear cosmid molecules. These recombinant molecules, prepared in vitro, are packaged in bacteriophage particles and transduced into an appropriate *E. coli* strain by selecting for a drug resistance marker present on the cosmid. This transformation gives rise to easily monitored recombinant clones that contain inserted genomic fragments in the 30–40 kilobase pair (kb) size range. For most procaryotic pathogens, this means that 200–500 transductant colonies likely will represent the entire genomic complement of the organism. This greatly simplifies finding the desired cloned fragment regardless of the screening method. Furthermore, a gene cloned in this fashion is recovered on a molecule that contains the regions of DNA that naturally flank it. This enhances the probability of obtaining genetic control regions with the cloned gene. The cloning of large fragments in cosmids also greatly facilitates the procedure of locating, by DNA-DNA hybridization, molecules that overlap with one another. Stepping from one overlapping cloned fragment to another enables one to "walk" along the chromosome and to gain valuable information on comparative gene positioning.

Disadvantages of cosmid cloning need to be pointed out as well. Large genomic fragments increase the probability of cloning a potentially lethal gene or group of genes in *E. coli*. Similarly, there is an increased chance of cloning fragments of genomic DNA that contain regions of unique structure; for example, stretches of DNA bordered by direct or inverted repeat sequences. Such regions in the cloned fragment might be excised and deleted even in recombinationless *E. coli* hosts, giving rise to misleading genomic clones.

An additional strategy for ensuring high yields of recombinant molecules containing both large and small inserted fragments involves the use of vectors that allows the direct selection of transformant clones that carry recombinant plasmids. Where tested, such vectors yield greater than 90% recombinant molecules in molecular cloning experiments. These vectors involve a variety of clever strategies that generally center on the cloned insert inactivating a plasmid gene that is inhibitory or lethal to the cell. Examples include lethal bacteriophage genes (126, 172), lethal endonucleases (14), and genes that, when inactivated, directly confer selectable drug resistance (25, 51, 118). Most

of these vectors are relatively new or not widely tested. However, our experience with one such vector, pOP203(A_2^+), constructed by Winter & Gold (172) indicates this type of vehicle to be quite efficient for the cloning of both large (30 kb) and small (2–4 kb) fragments.

Cloned procaryotic genes, more often than not, are transcribed and translated in *E. coli*. This expression enables one to screen for the desired transformant clone by looking for some new or altered physiologic trait or by immunologically probing colonies with antisera specific for the cloned gene product. Alternatively, if DNA probes are available (e.g. plasmids known to carry virulence genes), desired clones may be located by in situ colony DNA-DNA hybridization (90:312–26). Because of specificity inherent to the secretion of proteins by *E. coli*, one usually cannot count on foreign proteins being excreted from this organism even if this were known to be true in the host from which they were isolated.

Hence, immunologic screening of recombinant *E. coli* clones is usually accomplished by first disrupting colonies so as to release cloned gene products. If specific antisera to the antigen or suspected virulence protein being sought are available, immunologic screening can be accomplished in relatively convenient and sensitive ways (12, 183). To release cloned gene products, cells may be lysed chemically by relatively gentle means (134). Curtiss and coworkers (57) have used cells lysogenized with a thermoinducible λ phage to effect cell lysis. Proteins so released are able to react with specific antibody, forming a precipitin ring around the desired, lysed colony. Also, one of several phage λ cloning vectors may be used (90:269–93) and the formed plaques provide a sample of the lysed cell components that are detectable with immunologic reagents (183). The role of specific or nonspecific proteolysis in affecting the integrity of foreign proteins during phage-mediated lysis is not totally clear and may present problems in the immunological detection of such proteins. Although this is a possibility, thus far it does not appear to have hampered efforts to detect either large or small polypeptides.

The expression of cloned genes in *E. coli* may be evaluated conveniently by a variety of means. Aberrant cell division mutants of *E. coli* and *Bacillus subtilis* give rise to small anucleate cells called minicells (39, 115). Recombinant plasmids transferred by suitable genetic means to such mutant strains usually segregate into minicells. Such plasmid-containing minicells may be separated from vegetative cells and used to monitor plasmid-directed protein synthesis by incorporating radioactive amino acids into the protein. Alternatively, *E. coli* maxicell-producing mutants (122) may be employed. A recombinant plasmid-bearing strain carrying *rec*A *uvr* mutations is irradiated with ultraviolet light under conditions that result in chromosomal damage but leave intact a few plasmid molecules per cell. The chromosomal degradation that ensues leaves

the cell with only plasmid molecules that replicate and render the cell capable of synthesizing only plasmid-specific protein.

Chloramphenicol amplification (103) may also be employed to monitor plasmid-directed protein synthesis of cloned genes. Most cloning vectors continue to replicate in the absence of protein synthesis. Hence, chloramphenicol-treated cells accumulate hundreds of copies of plasmids. When such plasmid-amplified cells are washed free of chloramphenicol, selective expression of plasmid-linked genes may be followed for a finite amount of time. In all three of the aforementioned methods, radioactive proteins are monitored by autoradiography or fluorography of one- or two-dimensional polyacrylamide slab gels. The minicell method has enjoyed the widest use, owing to its relatively early discovery. However, the maxicell and chloramphenicol amplified cell methods offer the advantage of performing studies to localize cloned gene products in the vegetative cell. Neidhardt et al (102) indicate that the use of all three of these methods likely will greatly improve the chances of demonstrating the complete expression of a given cloned gene.

Although most work on the cloning of bacterial antigens and virulence determinants has used an *E. coli* host-vector system, other opportunities for both homologous and heterologous cloning exist. The operational cloning system in *Bacillus subtilis* has been useful in enhancing the exploration of cloned genes from gram-positive pathogens (30, 42). Considerable effort also has been made to develop host-vector recombinant DNA systems that use certain streptococcal species as hosts (87, 149). The prospects for direct cloning into other important pathogens such as *Haemophilus influenzae* (24), *Neisseria gonorrhoeae* (150), and *Staphylococcus aureus* (69) are also likely to be realized. Specialized systems for cloning in *Pseudomonas aeruginosa* also exist (4, 108, 174).

The natural transformability of certain species of pathogens makes the availability of "shuttle" cloning vectors highly desirable. Such vectors able to replicate and express drug resistance genes in both *E. coli* and the pathogenic host are useful in introducing cloned genes back into the homologous genetic background. Our laboratory has constructed shuttle plasmids able to replicate in *E. coli* and *S. sanguis* (88, 89). Shuttle vectors or potential shuttle vectors have also been reported for *S. pneumoniae* (5), *H. influenzae* (92), *S. aureus* (36, 50), and *N. gonorrhoeae* (150). In terms of extending cloning capabilities in a wide variety of gram-negative bacteria, Helinski and coworkers (28) have developed a binary plasmid system that facilitates the introduction of cloned genes into the host of origin by conjugation rather than by transformation. Fragments are cloned onto a broad host range plasmid that can be mobilized in *trans* by a conjugative plasmid that can only replicate in *E. coli*. This provides a means to introduce cloned genes back into the original host for physiological evaluation.

A potentially powerful application cited previously was the use of cloned sequences to obtain mutants in specific genes. To accomplish this, the pathogenic host must be amenable to some form of genetic exchange, preferably transformation or conjugation. Considering a situation in which a gene specifying a surface protein antigen has been cloned in *E. coli*, two possible strategies exist to obtain a mutant missing this antigen in the original host organism. First the gene is cloned onto a plasmid that is able to replicate in *E. coli* but not in the host of origin. A fragment containing a gene that is directly selectable in the host of origin is then inserted (e.g. transposed) or substituted in the coding sequence of the antigen gene. This plasmid can be mobilized by conjugation or transformed into the host of origin with selection for the newly inserted drug resistance gene. This resistance gene will be rescued by recombination, because it is flanked by sequences that are homologous with the intact chromosomal antigen gene. The integration event, however, interrupts the antigen gene, thus producing the desired mutant. Alternatively, the defective cloned gene containing the resistance marker can be purified and transformed directly into the host of origin with similar results.

As a second related strategy, the cloned antigen gene can be cleaved into subcistronic fragments using a suitable restriction endonuclease. These fragments are ligated to a plasmid able to replicate in *E. coli* but not in the host of origin. This plasmid must contain a resistance gene that is expressed in the host of origin. Hybrid plasmids constructed in this fashion are introduced, preferably by transformation, into the host of origin with selection for drug resistance. Because they contain only a portion of the antigen gene, their integration into the chromosomal homologue results in a small internal gene duplication along with an insertional inactivation. The simultaneous integration of the attached drug resistance gene makes this event, and consequently the isolation of the desired mutant, directly selectable. Methods similar to those described here have been used successfully in obtaining mutants of *Neisseria* (73, 74), *Bacillus* (37, 54), *Streptococcus* (162), and *Rhizobium* (120).

MOLECULAR CLONING IN GRAM-NEGATIVE BACTERIA

Escherchia Coli and Other Enteric Pathogens

Certain strains of the common intestinal inhabitant *E. coli*, called enterotoxigenic *E. coli* (ETEC), are capable of causing diarrheal disease in infants, adults, and young domestic animals (see 34). ETEC produce one or both of two types of enterotoxins. One such toxin, designated LT (heat labile toxin), is inactivated by heating, is immunogenic, and is composed of two subunits with estimated molecular weights of 25,500 (A subunit) and 11,500 (B subunit) (17,

21). The LT-A subunit causes the stimulation of adenyl cyclase in intestinal cells resulting in hypersecretion, while the LT-B subunit is believed to be involved in target cell binding (46). A second toxin, designated ST (heat stable toxin), is nonimmunogenic, thermostable, and has a M_r of 5,000 (1). There appear to be multiple types of STs, and one type is thought to exert its effects by stimulating guanylate cyclase (38, 61). In addition to enterotoxin production, ETEC possess proteinaceous, pilus-like surface antigens that facilitate colonization of intestinal epithelial cells by ETEC and enhance their ability to proliferate in the upper and/or lower small intestine of infected animals and humans.

As reviewed by Elwell & Shipley (34), these adherence antigens mediate mannose-resistant hemagglutination of human or animal erythrocytes, consist of protein subunits in the 20,000–30,000 M_r range, and are produced by cells grown only at temperatures above 18°C. These antigens tend to be host-specific. The porcine-specific antigen has been designated K88, and has been subdivided into three types: K88ab, K88ac, and K88ad. K99 appears to be specific for bovine and ovine hosts. Strains associated with human diarrheal disease may produce one of two specific adherence antigens termed CFAI or CFAII.

The enterotoxins and adherence antigens of ETEC are plasmid associated, a fact that has greatly facilitated their study. In virtually all cases these plasmids can be selftransmitted or mobilized by other conjugative plasmids. The experiments of Smith & Lingood (136) laid the foundation for understanding these virulence factors using isogenic strains made by conjugal transfer methods. The subsequent application of recombinant DNA methodology to this system has amplified greatly our understanding of the origin, expression, and dissemination of these virulence genes.

In 1976, So et al (139) reported the successful cloning of the gene specifying ST production from an *E. coli* of bovine origin. This was the first report of the cloning of a determinant concerned with bacterial virulence. The authors began with a 98-kb conjugative plasmid (ESF0041) that was known to carry the ST determinant. This ST determinant was cloned as part of an 8.9-kb *Eco*RI fragment from ESF0041. When this fragment was inserted into a multicopy plasmid the authors reported an approximate three-fold increase in ST production, consistent with an increase in gene dosage of the ST determinants. Further examination of this 8.9-kb fragment revealed that it contained a transposon-like structure consisting of an inverted repeat sequence of approximately 760 bp bordering an intervening region of about 530 bp (141, 142). Partial base sequence analysis and restriction endonuclease mapping provided compelling evidence that the inverted repeat sequence was the insertion element IS1.

The insertion of a fragment bearing a tetracycline resistance determinant into the intervening region resulted in the abolishment of ST production. By taking

advantage of the directly selectable tetracycline resistance marker, So and coworkers (141, 142) proved that the ST element was transposable. The ST transposon (Tn1618) was further characterized by insertion mutagenesis and by subcloning (142). Although repeated sequences have been associated with the structural genes of LT, transposing activity of this determinant has not yet been demonstrated (179). Direct base sequence analysis of the DNA bordered by the inverted repeat sequences revealed the presumed ST gene to possess the needed transcriptional and translational start and stop signals as well as a 16S ribosomal binding site. The deduced amino acid sequence specified by the ST gene yielded a protein of approximately 8000 M_r. This value was considerably larger than the previously published value of 5000. So and McCarthy (142) postulated that this discrepancy was due to the presence of an amino terminal "signal sequence" on the ST peptide needed in the transport of the toxin through the cell membrane. Such signal peptides are proteolytically removed from the molecule during transport. Thirteen of the first eighteen amino acids at the amino terminal end of the peptide were hydrophobic, consistent with signal sequence composition. In fact, the first four amino acids of the ST peptide were identical to those found in the signal sequence of the bacteriophage fd minor coat protein. The precise definition of the postulated ST signal sequence will require further work.

Biological and biochemical data provided evidence that ST molecules from different ETEC strains represented a heterogeneous group (13, 52). ST I (also called STa) is soluble in methanol and is reactive in an infant mouse model system. So and coworkers (139, 141) cloned an ST of this type, designated ST Ia. ST II is insoluble in methanol and is reactive in a ligated pig ileal loop model but not in the infant mouse model. Exploitation of molecular cloning methods has contributed to our understanding of the differences among ST determinants. First, DNA-DNA hybridization studies using a ST Ia probe revealed this sequence to be disseminated in strains of human, bovine and porcine origin (97, 99, 138). A second ST I gene, designated ST Ib, has been cloned and its base sequence determined (99). The ST Ia and ST Ib genes display a sequence divergence of 31%.

Despite this divergence, structural similarities between the two amino acid sequences exist. The amino terminal ends of both molecules are rich in hydrophobic amino acids, suggesting the presence of signal peptides. The number and positioning of cysteine residues in the two molecules are remarkably similar. Finally a stretch of some 18 amino acids near the carboxy-terminal end of both molecules is highly conserved. Based on this striking homology in amino acid sequence, Moseley et al (99) suggested that this region may play a crucial role in the functioning of the toxin. These authors have used both ST Ia and ST Ib sequences as molecular probes in screening stools from patients with diarrhea for the presence of ETEC. Most strains recovered from such stool

specimens contained ST Ib–hybridizing sequences and a few showed hybridizing activity to both probes—indicating the presence of both ST gene types. Further epidemiological studies by Moseley et al (100) have revealed genes homologous with the ST Ia and ST Ib probes present on two different plasmids in the same ETEC isolate of human origin. These findings confirmed the genetic and serological data suggesting the presence of two ST genes in this particular strain (180).

Recently, two groups have cloned and sequenced the gene for ST II (81, 111). Both groups arrived at the same sequence using ST-plasmids of different origin. Interestingly, neither the DNA or amino acid sequence of the ST II gene displayed any resemblance to the sequenced ST I genes. However, peptide size in both cases was remarkable: 71 amino acids for ST II vs. 72 amino acids for ST I. The differences seen between the structure of the ST I and ST II molecules were consistent with their biological properties. ST I is active in the infant mouse model, while ST II is active in the pig jejunal loop model. In addition, although ST I is recognized as stimulating guanylate cyclase, the precise role of ST II in pathogenicity is not yet known (13). The occurrence of the identical ST II gene on two temporally and geographically distinct plasmids is in contrast to the situation seen thus far with the ST I genes. The latter have displayed evolutionary divergence (81, 97, 111), whereas the former are rigidly conserved. Although the highly conserved ST II gene on different plasmids is suggestive of the notion that this gene might be part of a transposable element, no supportive evidence is yet available.

So et al (140) first cloned fragments specifying LT activity from a 90-kb plasmid of porcine origin. Ultimately these authors were able to trace LT production to a single *Bam* HI fragment, 8.9 kb in size. Using *E. coli* minicells and restriction endonuclease–generated deletion formation, Falkow and colleagues further characterized the LT-specifying cloned fragment (21, 23) and were able to identify a region of about 1.9 kb associated with LT production. This region was found to encode proteins of 11,500 and 25,500 daltons in *E. coli* minicells. The smaller protein (designated the B subunit) adsorbed to Y-1 adrenal cells and was shown to form aggregates consisting of 4–5 monomers. The 25,500 M_r peptide (designated the A subunit) was found to have adenylate cyclase–activating activity. Deletion mapping data enabled these workers to propose relative locations of the two structural genes encoding LT activity (*elt*A [subunit A] and *elt*B [subunit B]). Certain deletions also suggested that both structural genes were transcribed from a single promoter (*elt*A to *elt*B). Dallas & Falkow (22) subsequently have determined the nucleotide base sequence of the LT-B specifying gene (*elt*B). These data did not reveal any type of promoter sequence immediately upstream from the *elt*B gene; however, a 16S ribosomal binding site was seen 3 nucleotides from the *elt*B initiation codon. This provided additional support that the *elt*A and *elt*B genes are

transcribed as a single polycistronic message from a promoter proximal to *elt*A. The *elt*B sequence data also led Dallas & Falkow (22) to postulate the existence of a 21 amino acid signal sequence.

Yamamoto's group has cloned sequences specifying LT activity from an ETEC of human origin and reached similar conclusions relative to human LT gene organization and expression (178, 182). Their base sequence analysis supported the presence of a single polycistronic message but further revealed that the A subunit gene (called *tox*A for the human toxin) overlaps the B subunit gene (*tox*B) (177, 181). Their data indicate that the *tox*A stop codon shares two common bases with the start codon of the *tox*B start codon. Hence the same ribosome is predicted to translate the two adjacent cistrons. This provides some explanation as to why the *tox*B gene product is produced in excess of the *tox*A gene product. The 16S ribosome binding site seen by Dallas & Falkow (22) near the *elt*B (porcine version of *tox*B) also has been invoked to explain this observation (20, 177).

Cloned LT determinants from ETEC of human and porcine origin have afforded direct comparison of their relatedness. Using restriction endonuclease mapping and DNA-DNA hybridization, Dallas (20) concluded that these determinants had strikingly similar restriction maps and that they were highly homologous within the toxin coding region. The DNA flanking the respective determinants was nonhomologous, however. Yamamoto's group (176) expanded these observations by determining a partial nucleotide sequence of the human LT-B gene (*tox*B) and comparing it to the sequence of its porcine counterpart (*elt*B) as reported by Dallas & Falkow (22). The genes were found to be 97% homologous at the nucleotide level (90% homologous at the amino acid level) in the region encoding the putative signal sequence. Only one amino acid change was noted in the first eight positions of the putative mature gene product. The remainder of the human *tox*B gene will have to be sequenced to complete the comparison, but the initial data indicate high homology. Additional cloning efforts by Yamamoto and co workers (182) have led to the construction of plasmids that specify just the product of the *tox*B gene, which in the absence of the *tox*A product is not toxic. Recombinant plasmids that specify a truncated *tox*A gene product, which is immunoreactive but nontoxic, have also been produced (182). These toxoids should have potential as vaccine candidates.

The LT activity of ETEC and the enterotoxin of *Vibrio cholerae* display remarkable similarity (23). Both are bicomponent in nature (subunit A = adenyl cyclase–stimulating activity, subunit B = target cell–binding activity), are similar in molecular mass, and share immunologically cross-reactive determinants. Considerable molecular evidence is now available to support the relatedness of LT and cholera toxin (CT) (22, 98, 144). DNA-DNA hybridization studies revealed the *elt*A and *elt*B genes to be on the order of 70%

homologous with sequences on the *V. cholerae* genome (98). These in situ hybridization studies also suggested that the hybridizing sequences did not always occupy the same site on the *V. cholerae* genome and that in some cases these sequences were present to the extent of multiple copies per chromosome.

On the other hand, the hybridization patterns with the separate *elt*A and *elt*B probes indicated that the A and B subunit genes were linked in *V. cholerae*. The amino acid sequence of the *elt*A and *elt*B gene products deduced from the nucleotide base sequence has been compared to the available amino acid sequence data for CT (22, 144). The target cell binding activity (*elt*B, CT-B) from each organism showed a high degree of relatedness, 79% at the amino acid level. The *elt*A nucleotide base sequence (144) has also been compared to available amino acid sequence data for CT-A. Homology between the activities from the two sources was evident but varied within the molecule (40–79%). The *elt*A sequence data also suggested the presence of an 18–amino acid signal sequence. Because about 35% of the amino acid sequence of CT-A currently is available, a full comparison of it to the *elt*A gene product cannot yet be made.

Availability of the CT-A and -B genes on recombinant plasmids will facilitate the thorough comparison between ETEC and *Vibrio cholerae* enterotoxins. A preliminary report of the successful cloning of CT genes appeared in 1981 (65). Detailed reports of cloning CT genes in two different laboratories appeared in 1982 (45, 110). Pearson & Mekalanos (110) used ETEC-cloned LT probes to detect CT genes in a genomic library prepared in *E. coli*. They observed the CT-A and CT-B genes to be linked and, using restriction mapping data, verified that the CT and LT cistrons were quite similar in organization. The CT produced in *E. coli* was found to be largely cell-associated, indicating that this host was unable to secrete the toxin in contrast to *V. cholerae*.

Gennaro & Greenaway (44) have begun the determination of the nucleotide sequence of the CT genes. Sequence data have been used to generate the amino and carboxy termini of the A subunit and the amino terminus of the B subunit of CT. They postulate that both the CT-A and CT-B peptides contain hydrophobic signal sequences that are cleaved off as the molecules are secreted through the cell membrane. Genetic regulatory features reminiscent of the ETEC LT genes also were found: (*a*) no intercistronic region existed between the A and B genes; and (*b*) a 16S ribosomal binding site was observed six nucleotides upstream of the start of the CT-B coding sequences. Nucleotide sequence comparison of the regions corresponding to the putative signal peptides and amino termini of the mature CT-A and human LT-A (*tox*A) toxins revealed 70–75% homology. Similar comparisons using the same regions of the B subunits of both toxins showed 58% homology. As the complete sequence of the CT genes becomes available, a rigorous comparison can be made. These studies and the tools they generate also are likely to lead to an understanding of how these genes became disseminated in such distantly related bacteria.

The genes for the related K88ab and K88ac adherence antigens have been cloned from large K88-plasmids and studied using deletion mutagenesis, transposon mutagenesis, nucleotide sequence analysis, *trans*-complementation, and minicell-directed protein synthesis (29, 41, 67, 68, 95, 96, 128). A minimum of four cistrons are involved in K88ac synthesis and this is likely the case for the K88ab determinant as well. Kehoe et al (67) have proposed for K88ac that the determinant is composed of two operons. One operon is believed to contain the structural gene for the K88ac antigen subunit (*adh*D). A second operon is thought to encode three genes: one (*adh*A), and possibly a second (*adh*B), involved in pilus assembly, and a third hypothesized to be a positive regulator of *adh*D.

All of the gene products made by these cistrons appear to be synthesized as precursor molecules that ultimately are processed to remove putative signal sequences (68). Similar results have been observed for at least two of the polypeptides encoded by the K88ab determinant (96). Direct base sequence analysis of the K88ab antigen gene (equivalent to *adh*D) revealed a signal peptide of 21 amino acids (41). These sequence data also showed that K88ab was unrelated to the K99 and CFAI antigens at the amino acid level based on partial sequence data available for the latter two. The issue of transcriptional control of the K88 operons is not yet fully resolved. Recently Kehoe et al (68) have reported the identification of a fifth gene (*adh*E) that is located at the proximal end of the K88ac determinant and seems to be required for high level expression of K88ac pili. The K99 determinant also has been cloned in *E. coli* using recombinant DNA methods (62, 159). Initial studies on the genetic organization and expression of the K99 determinant indicate a pattern similar to K88: five cistrons divided into two operons, with the pilus subunit determinant being under positive control (Isaacson, submitted for publication). The cloning of human CFAI has proved difficult but recently Rowe and coworkers (171) have succeeded in accomplishing this task. The expression of CFAI in *E. coli* required that two separate regions of a large CFAI plasmid be cloned separately on compatible plasmids and introduced into the same cell.

The study of adherence factors of *E. coli* has led to the exploration of unique strains of this organism that are associated with urinary tract infection in humans. Two specific types of adherence determinants, type 1 pili (mediate mannose-sensitive hemagglutination) and P, or *pip*, pili (mediate mannose-resistant hemagglutination) are recognized as being important in facilitating adherence of these strains to epithelial cells of the urogenital tract. These pili, especially the *pip* types, are believed to be virulence factors (64, 157). Determinants of both types have been cloned in *E. coli*, and intensive study is underway (type 1 cloning, see 58; *pip* pili cloning, see 16, 58, 116, 158). Unlike the adherence antigens involved in *E. coli*-mediated enteric disease, these determinants are chromosomally located. The two pilus types have been

found to be genetically distinct and are able to confer the appropriate adhesive properties in *E. coli*. A cloned fragment expressing the *pip* pili phenotype was found to specify multiple polypeptides, among them the 19.5-kdal *pip* pilin subunit (107). The nucleotide sequence of the entire *pip* pilin subunit cistron (*pip*A) recently has been determined (Baga et al, in preparation). Its amino acid sequence homology is different from the K88 antigen but the molecules share a similar hydrophobicity profile. Genes encoding *Klebsiella pneumoniae* type 1 pili also have been cloned (113). The availability of cloned adhesin genes from *E. coli* and *K. pneumoniae* should afford the opportunity to focus clearly on the role of these characteristics to pathogenicity using in vivo models. Equally important, cloned adhesins provide the materials for the high-resolution molecular analysis of their gene organization and expression.

Another class of surface antigens that have come under study using recombinant DNA methods are the flagellar proteins (flagellins) of the enteric pathogen *Salmonella typhimurium*. By cloning such genes, Simon and coworkers have elucidated a unique control system that governs the expression of the two flagellin genes (H1 and H2) of *S. typhimurum*. Only one of these genes is expressed at any time, so the cell makes either H1- or H2-type flagella, but not both. The ability to shift this antigenic expression is called phase variation. Cloned versions of the H2 gene in the "on" and "off" position revealed that the H2 gene existed next to a 995-bp sequence that was invertable via a site-specific recombination event (131, 133, 184). This region contained a promoter for the H2 gene (133, 185) that also appeared to cotranscribe an H2-adjacent gene called *rhl* (132). It has been postulated that *rhl* is a repressor protein controlling the expression of the H1 gene (132). The regulatory role of the *rhl*-specified polypeptide (thought to be 16 Kdal) has not yet been established, however.

Thus, in one orientation the 995 bp region results in the expression of the H2 gene with concomitant repression of the H1 gene, owing to *rhl* transcription and translation. In the opposite orientation, neither H2 flagellin nor H1 repressor (proposed *rhl* gene product) are transcribed, allowing the H1 flagellin gene to be expressed. The 995 bp region also contains a gene (*hin*) that specifies a 19-Kdal polypeptide implicated in the inversion event (153). Partial sequence analysis of the H1, H2, and the cloned *E. coli* flagellin gene, *hag*, has revealed these genes to be highly homologous at their amino-terminal ends (153). All three genes also showed significant regions of homology upstream from their start codons. The H1 and *hag* genes were found to contain sequences that could specify a polypeptide that consisted of the same 9 amino-terminal amino acids (*rfs* gene). Sequence data beyond that limit are not available. However, these data suggest that the control mechanisms for flagellin gene transcription and translation shared a common ancestry. Genetic and biochemical evidence currently indicate that both the *pip* pili and type 1 pili of *E. coli* also undergo

phase variation (33, 117). The appropriate cloned versions of these genes should provide an answer to the basis of such pilus regulation using the approaches employed by Simon and colleagues.

Molecular cloning has greatly facilitated our understanding of the role that α-hemolysin production plays in the virulence of *E. coli* strains involved in extraintestinal infections. The lysis of mammalian erythrocytes by bacteria generally is thought to increase virulence, and *E. coli* strains that are hemolytic are related epidemiologically to extraintestinal infection (59). Such hemolytic activity may reflect a generalized ability to perturb mammalian cell membranes and their associated functions, thereby exerting pathologic changes. Using classical transmission genetics and gene cloning methods, hemolysin determinants *(hly)* have been demonstrated on different plasmids and on the chromosome of *E. coli* (26, 48, 135, 168). *E. coli hly* determinants appear to be closely related when measured by nucleic acid hybridization (27, 101, 168). Early experiments aimed at assessing the role of plasmid-linked *hly* determinants in virulence involved transferring large, conjugative *hly* plasmids into nonhemolytic strains and testing such strains using animal models (83, 164). Although such types of studies implicated *hly* as a virulence factor, the possibility that unrecognized plasmid genes were contributing to virulence could not be discounted. This problem was resolved when Welch et al (166) cloned a *hly* determinant as an 11.7-kb DNA fragment and, using transposon mutagenesis, localized the *hly* determinant to a 7.5-kb region. Strains containing the cloned *hly* sequence or a transposon-inactivated version have been tested in a rat peritonitis model and clearly show that the *hly* determinant confers virulence in *E. coli*. Goebel and coworkers (47, 53) have isolated a *hly* determinant from the naturally occurring *hly* plasmid pHly 152 using a sequential cloning strategy. Deletion mapping studies indicate the entire determinant occurs over a stretch of 6–7 kb. Employing an approach similar to that taken by Welch et al (166), these workers demonstrated *hly*-associated virulence also by using cloned *hly* genes (53).

Recent studies have focused on differences seen among various cloned *hly* determinants with respect to virulence contribution (referred to as toxicity by Goebel and colleagues). Reasons for these observations center on small nucleotide sequence differences among the various determinants (53) and on differences in the genetic regulation and secretion of the hemolysin (167). Further work is needed to resolve this issue, but the two possibilities are not mutually exclusive (167). The cistronic structure of the *E. coli hly* determinants is being studied using these cloned genes in connection with deletion and transposon mapping, *trans*-complementation, and minicell-directed protein synthesis (47, 104, 105, 148, 165). Multiple cistrons clearly are involved, including genes for the hemolysin precursor, transport, and, possibly, activation functions.

Other virulence factors recognized in *E. coli* also are undergoing study using recombinant DNA methods. Resistance to the bactericidal effects of serum has been suggested to confer virulence (2, 31). A 5.3-kb fragment cloned from the *Col*V-I94 plasmid was found to specify the serum resistance phenotype and enhanced virulence in *E. coli* KH933 (7). *E. coli* K-12 bearing the cloned determinant displayed increased survival in serum, but did not display an increase in virulence. A similar activity has been cloned from the R6-5 plasmid and localized to a region of about 600 bp (94). The gene product specified by this gene has been identified as the *tra*T protein and is known to be a major outer membrane protein that mediates plasmid surface exclusion. High and low copy recombinant plasmids containing just this gene or its *Col*V-I94 counterpart should be readily attainable and will be useful in evaluating the regulation and precise contribution to virulence of this class of genetic information.

Plasmid *Col*V-K30–linked genes specifying an efficient iron-sequestering mechanism have been associated with virulence in mice (170). Recently, a 16-kb fragment specifying a high-affinity iron assimilation system has been cloned from the *Col*V-K30 plasmid (6). This accomplishment should greatly facilitate our understanding of the number, organization, and regulation of expression of the genes by iron. In addition, because virulence testing thus far has involved large plasmids or their mutants, the localization of the iron-sequestering determinant will provide better means for virulence evaluation.

Production of K1 polysaccharide capsule enhances *E. coli* virulence, probably by exerting an antiphagocytic effect (124). Silver et al (130; Silver et al, submitted for publication) and Echarti et al (32) have succeeded in cloning the genes of K1 capsule biosynthesis. A picture of the organization and function of genes on these cloned determinants is emerging (32; Silver et al, submitted for publication). The K1 capsular genes were not able to enhance the invasiveness of *E. coli* K-12 in preliminary experiments using a rat model.

Neisseria and Haemophilus

Neisseria gonorrhoeae surface pili are composed of an 18-kd protein subunit, and they represent a major surface antigenic determinant. Pili are responsible for gonococcal attachment to epithelial cells and their presence is associated with virulence (152). Piliated strains give rise to revertable, nonpiliated ones. Meyer et al (93) successfully cloned the structural gene for the 18-kdal pilin gene from *N. gonorrhoeae*. The gene product could be detected immunologically on the surface of *E. coli* cells but was not assembled into recognizable pili. Using the cloned pilus gene as a hybridization probe, the authors presented evidence indicating that the transition to and from the piliated state involved a specific, reversible rearrangement of chromosomal genes. The availability of the cloned pilin gene makes the elucidation of this apparent recombinational control mechanism approachable. In addition, the experiments of Meyer et al (93) underscore the feasibility of developing vaccines for gonorrhea using purified pili. A preliminary report of cloned gonococcal pilus genes from a different strain has also appeared (75). Cannon's group (8, 9) has cloned a gene specifying the gonococcal H8 outer membrane protein, an antigen common to pathogenic *Neisseria*. This cloned determinant has been used successfully to assay human immune response to the H8 antigen in patients with disseminated gonococcal disease and in patients with meningococcemia.

N. gonorrhoeae and *Haemophilus influenzae* number among several pathogenic species of bacteria that are known to produce an extracellular protease that cleaves specifically secretory immunoglobulin A1 (sIgA1; 112). These proteases are regarded as virulence factors for obvious reasons, but rigorous testing of this assertion is not yet available. Koomey et al (73) have cloned the sIgA1 protease from *N. gonorrhoeae* F62. Enzyme activity in *E. coli* was greatly depressed compared to *N. gonorrhoeae* (0.5–2%) and a 140-kd polypeptide specified by recombinant plasmid-containing minicells correlated with sIgA1 protease expression. These authors removed a 1.8-kb fragment from within the sIgA1 protease coding fragment and replaced it with a fragment containing a directly selectable ß-lactamase gene. When the purified fragment containing the disrupted sIgA1 gene was used to transform *N. gonorrhoeae*, ampicillin-resistant clones were found to be sIgA1 protease–deficient.

Recently (11) the sIgA1 protease of *H. influenzae* has been cloned in *E. coli*. The sIgA1 protease coding region of the cloned fragment has been localized to 3.5-kb DNA fragment, and a 137-kd polypeptide, presumed to be the sIgA1 protease, has been identified in *E. coli*. A portion of the cloned *H. influenzae* determinant was used as a probe to demonstrate the occurrence of homologous sequences in serogroups a–f of *H. influenzae*. Hybridizing sequences were not found in an sIgA1 protease–negative, nonpathogenic *H. parainfluenzae*. Extending this molecular epidemiology approach, Koomey & Falkow (72a) have demonstrated that the sIgA1 protease gene from *N. gonorrhoeae* shares homology (67–75% range) with genomic DNA from *N. meningitidis* and surprisingly, from *H. influenzae*. Thus, despite their significant evolutionary divergence, *Neisseria* and *Haemophilus* share a related sIgA1 protease gene. The exact nature of the relatedness of the *Neisseria* and *Haemophilus* sIgA1 protease genes can now be obtained by nucleotide base sequence analysis.

Pseudomonas

Pseudomonas aeruginosa, an important cause of infections in compromised hosts, possesses a variety of recognized extracellular virulence factors (84). These include hemolysins, proteases, and a potent toxin, exotoxin A. The use of mutant strains has aided our understanding of virulence in this organism (10), but the cloning of presumed virulence factors has proceeded slowly. The molecular cloning of the phosphate-regulated hemolysin gene (a phospholipase

C activity) was first reported by Vasil et al (161). The phospholipase C activity largely was cell-associated in *E. coli* in contrast to *P. aeruginosa*. The cloned fragment was introduced into a *P. aeruginosa* host using an appropriate vector, and extracellular proteins were monitored by polyacrylamide gel electrophoresis. A 78-kdal polypeptide, consistent with phospholipase C, was found in elevated amounts along with five other proteins in the 28–37-kdal range. These proteins were assumed to be encoded on the cloned 6.2-kb fragment and may be part of a regulated phosphate transport system.

Other groups (18, 86) since have cloned a phospholipase activity from *P. aeruginosa* that appears identical to the one reported by Vasil et al (161). The 78–80 kdal phosphalipase C protein is made in *E. coli* minicells and maxicells (18, 86). The question of whether a vector promoter (i.e. that of tetracycline resistance gene) is contributing to transcription of the cloned phospholipase C gene needs to be resolved. The isolation of large fragments from *P. aeruginosa* by cloning in *E. coli* has been difficult for reasons that remain unclear (18). Pseudomonad genes often do not appear to be well expressed in *E. coli* (Chakrabarty, personal communication). The cloned phospholipase gene should provide a departure point for studying such problems at the molecular level as well as providing assistance in evaluating the importance of this virulence factor.

MOLECULAR CLONING IN GRAM-POSITIVE BACTERIA

Streptococcus

The streptococci are under intense study using recombinant DNA methodology. Streptococcal genes appear to be well expressed in *E. coli*, and a variety of biological activities have been cloned that are not directly or obviously associated with virulence (55, 63, 137). These will not be discussed in this review. Curtiss et al and Robeson et al (19, 119) have cloned a determinant from a cariogenic *S. mutans* that expresses glucosyl-transferase activity in *E. coli*. Glucosyl transferase–mediated synthesis of water-insoluble glucans by *S. mutans* is believed to be crucial in caries formation (40, 154). The role of the cloned glucosyl-transferase activity group in glucan synthesis in *S. mutans* currently is under study.

The enzymatic activity cloned in *E. coli* has a number of unique features. Compared to native glucosyl-transferase activity from *S. mutans*, the cloned activity is smaller in molecular size [55-kdal polypeptide in minicells vs ~150 kdal for native enzymes (see 79)], has an associated glucanase activity, and was not stimulated by the addition of exogenous glucan. *E. coli* strains carrying this determinant synthesized a glucan of 1,500 M_r, but the nature of its glycosidic linkages are not yet known. Use of this cloned sequence to do site-directed mutagenesis should enable the assessment of this particular determinant's contribution to glucan synthesis and virulence in *S. mutans*. Curtiss's group also has successfully (57) cloned in *E. coli* sequences that specify *S. mutans* surface proteins. This work sets the stage for the assessment of antigens that may play a role in adherence of *S. mutans* and in the development of anticaries vaccines.

Streptococcus pyogenes (Lancefield group A) is an important human pathogen that produces a fibrillar surface antigen, called M protein, that mediates an antiphagocytic activity. The organism is able to vary the structure of M protein from an immunologic standpoint, and numerous different types of M-proteins (>70) are known. Scott & Fischetti (127) have reported the successful cloning of the structural gene for type M6 protein from *S. pyogenes* D471. Using immunoblot analysis, *E. coli* was found to make three polypeptides with antigenic deteminants identical to M6 protein. The reasons for multiply sized M6 reactive polypeptides seen in *E. coli* are not known, but all three were larger than M6-reactive polypeptides extracted from streptococcal cells. Preliminary reports of other types of streptococcal M proteins are emerging (M24:12a; M5, M19:112a). Lack of efficient means (i.e. transformation) to reintroduce cloned genes back into *S. pyogenes* will hamper the study of the relative contribution of virulence factors in this pathogen. Systems involving conjugal transfer and/or transduction that operate in group A streptococci may be helpful, however. On the other hand, this streptococcal cloning can be expected to open up some interesting possibilities for the development of cell-free streptococcal vaccines using cell products prepared in *E. coli*. Malke & Ferretti (89a) recently have cloned in *E. coli* the gene specifying streptokinase (a plasminogen activator) from *S. equisimilis* (Lancefield group C). This protein is produced and exported by *E. coli*, making the production of this clinically utilized enzyme possible using nonstreptococcal sources. Obviously this cloned gene can now be employed as well in studies aimed at evaluating its contribution in pyogenic streptococcal infections.

A final example of recombinant DNA methods applied to potential streptococcal virulence involves the recent work of LeBlanc et al (80), who have cloned streptococcal plasmid fragments that are known to correspond to parts of an hemolysin-bacteriocin *(hly-bcn)* gene. These fragments were used as molecular probes and revealed that this determinant was disseminated on plasmids from *S. faecalis var. zymogenes* from five different geographical locations. However, this disseminated, plasmid-linked hemolysin determinant did not show homology to DNA from ß-hemolytic streptococci representing Lancefield group A, B, F, and H.

Staphylococcus

Several genes suspected to be important in the virulence of *Staphylococcus aureus* have been cloned. The structural gene for staphylococcal protein A, an immunoglobulin-binding polypeptide, has been cloned in *E. coli* and partially

sequenced (85). By conventional amino acid analysis, protein A is known to contain four repetitive domains that elicited immunoglobulin Fc binding. The amino acid sequence data obtained from the nucleotide sequence determination indicated yet a fifth such binding site as well as the presence of a putative signal sequence. The cloned protein A was not secreted by *E. coli* cells. Staphylokinase, a blood plasminogen activator, is produced by *S. aureus* strains lysogenized with bacteriophage SØ-C. This was established by Sako et al (121), who cloned the staphylokinase structural gene on a DNA fragment from SØ-C.

A gene encoding the staplylococcal α-hemolysin has been cloned in *E. coli* and genetically defined using restriction mapping and transposon mutagenesis (66). This cloned determinant has been introduced into *B. subtilis* and *S. aureus* using appropriate shuttle plasmids (36) and the comparative expression of this determinant has been studied. The cloned α-hemolysin was secreted poorly in *E. coli*. However, this cloned determinant conferred hemolysis in *B. subtilis* and in nonhemolytic mutants of *S. aureus*, indicating expression and gene product secretion in these organisms.

Recently, the gene encoding the unique exotoxin produced by *S. aureus* strains associated with toxic shock syndrome has been cloned in *E. coli* (77). Expression of this exotoxin could be monitored immunologically in *E. coli*, where it was found to exist as a periplasmic protein. Sub-cloning experiments indicated the coding region of this gene was present on a 300 base pair fragment. Using the cloned gene as a hybridization probe, these workers showed that naturally occurring, exotoxin-nonproducing strains were missing the gene or contained it as part of sequences that were rearranged relative to exotoxin-producing strains. The possibility that this gene may be turned "on" or "off" by strains regulating virulence can and should be explored by cloning and studied by comparing sequences bearing the putative inactive form of the exotoxin gene.

Bacillus

Strains of *Bacillus thuringiensis* produce crystalline inclusion bodies during sporulation. These crystals are composed of a 130-kdal polypeptide called the crystal protein or delta-endotoxin. Depending on the subspecies of *B. thuringiensis*, these crystal proteins are toxic for lepidopteran or dipteran insects. Preparations of spores and crystals are utilized commercially as insecticides. The structural gene for crystal protein has been cloned from three different *B. thuringiensis* strains (56, 71, 125). The cloned gene makes a polypeptide of similar size in *E. coli* cells. Protein extracts of *E. coli* cells bearing the active cloned gene were toxic to tobacco hornworm larvae. The cloned gene has been used as a probe to establish the plasmid linkage of the crystal protein cistron (56, 71. 125). This notion has been further supported by conjugal transmission studies (49). Using a combination of conjugal transmission and cloning

approaches, strains of *B. thurigiensis* have been constructed that produce two different crystal proteins, one with lepidopteran specificity and one with dipteran specificity (70). The existence of chromosomal copies of the crystal protein gene, which may be genetically inactive, have been postulated (56, 70, 71).

Recent work by Kronstad et al (78) indicates a common crystal protein gene to be widely disseminated on plasmids in different *B. thuringiensis* strains; some strains contained multiple gene copies of the crystal protein gene. Only 1 out of 22 strains was found to have a crystal protein gene of implied chromosomal origin. Nucleotide sequence analyses of two of the cloned crystal protein genes are underway (15, 72). A gene encoding a mosquito larval toxin from *Bacillus sphaericus* also has been cloned and expressed in *E. coli* (43). The cloning of insecticidal proteins from pathogenic bacilli promises to be rewarding especially in terms of the construction of more effective specific pathogens for use in naturally controlling insect populations.

The so-called protective antigen of the important veterinary and human pathogen *Bacillus anthracis* has been cloned in *E. coli* by Vodkin & Leppla (163). Expression of the *B. anthracis* protective antigen gene was identified by immunoblotting, and its biological activity was demonstrated using an in vitro Chinese hamster ovary cell elongation assay. Because the protective antigen of *B. anthracis* is believed to induce a protective immunity to anthrax, these cloning experiments should open avenues to develop safer, more effective vaccines to control anthrax.

A sequence that specifies a thio-activated hemolysin from *Bacillus cereus* has been cloned in *E. coli* and moved via shuttle plasmid to *B. subtilis* (76). Although apparently weakly expressed in *E. coli*, this gene converted *B. subtilis* to a hemolytic phenotype. The cloned gene did not appear to hybridize to chromosomal DNA from bacteria that also made thio-activated hemolysins (*S. pyogenes, Listeria monocytogenes*). Further comparison of this class of hemolysin determinants at the nucleotide sequence level is warranted because such toxins are related biochemically and immunologically.

Corynebacterium

The toxin produced by *Corynebacterium diphtheriae* is encoded by a gene carried on bacteriophages that lysogenize this organism (109). The diphtheria toxin (62 kdal) can be separated into two fragments by trypsin digestion. The A fragment inactivates eucaryotic elongation factor 2 via adenosine diphosphoribosyl transferase (ADRT) activity. The B fragment mediates a cell-binding activity and possibly the passage of the toxin through the target cell membrane. Leong et al (82) cloned an 831-bp *Sau* 3A corynephage fragment that encoded the amino-terminal end of the toxin, including the signal sequence and the A fragment–encoding region. *E. coli* cells carrying this sequence had periplasmic

ADRT activity that was inhibited by antidiphtheria antibodies. Recent work by Leong et al (submitted for publication) has shown that the cloned sequences encode a protein that, when trypsin treated, gives rise to polypeptide presumed to be the A fragment. Evidence that the *E. coli* secretory apparatus transports the cloned protein to the periplasm has been obtained using *sec*A mutants. This suggests that signal sequences from proteins of gram-positive organisms can be recognized and processed by *E. coli*. Tweten & Collier (156) have also successfully cloned in *E. coli* a phage sequence encoding the A fragment of the toxin. In addition, they have cloned a second sequence that spans the A fragment region plus about 60% of the B fragment region. Regulatory and coding sequences of the diphtheria toxin gene recently have been identified at the nucleotide base sequence level (114). The molecular cloning of the diphtheria toxin provides an important step towards the development of better vaccines, the creation of "targeted toxin" molecules (3), and the comparative study of bacterial secretory mechanisms.

MOLECULAR CLONING IN UNUSUAL PATHOGENS

The study of pathogenic microorganisms that are difficult, if not impossible, to cultivate in vitro can be facilitated by using recombinant DNA methods. Bassford and coworkers (145–147) were the first to report the cloning in *E. coli* of genes from the etiologic agent of syphilis, *Treponema pallidum*. These workers now have identified in recombinant *E. coli* a number of immunoreactive polypeptides believed to be surface antigens of *T. pallidum*. IgG antibodies to some of these proteins have been detected in rabbits that have been experimentally infected with *T. pallidum*. Van Embden et al (160) and Norgard & Miller (106) subsequently have reported the successful cloning and expression of *T. pallidum* genes in *E. coli*. The availability of cloned *T. pallidum* genes should assist our understanding of antigens that are important to the host protective response and perhaps in *T. pallidum* virulence. Obviously the identification of polypeptide immunogens for use as vaccines is a central goal of such work. The development of more useful diagnostic reagents also should guide such research efforts.

Work is also underway with other unusual or difficult-to-cultivate pathogenic bacteria. A gene specifying a 19-kdal antigen from the obligate intracellular pathogen *Chlamydia trachomatis* has been cloned and is expressed in *E. coli* (169). A second chlamydial antigen (74-kdal) gene has also been cloned (151). *Legionella pneumophila* genes that express immunoreactive proteins have been reported in preliminary form (35). Cloned genomic DNA from *Mycoplasma hyorhinis* also has been found to be transcribed and translated in *E. coli* (155). Finally, a sequence cloned from chromosomal DNA of *Rickettsia prowazekii* has been reported to express functionally a citrate synthase activity in *E. coli* (173). This work should open avenues to the study of *Rickettsia* genes that may be involved in antigenicity and virulence.

CONCLUDING REMARKS

Recombinant DNA methodology has added a new dimension by which pathogenic bacteria may be studied. Cloned sequences enable us to ask questions about virulence genes that would otherwise be virtually impossible to address. The next several years will likely see some very significant advances in this field. The macromolecular fine structure of toxins and its implications for toxic activity and/or target cell binding should be an area of rapid development. A more complete understanding of how protein secretion operates, especially in an intergeneric, comparative sense is likely to emerge as well from studies on cloned toxins or antigens. The use of cloned genes to induce specific mutations in pathogenic bacteria probably will be employed widely as a primary strategy for assessing the contribution of specific genes to colonization and virulence. Finally, the use of cloned gene products for the development of immunogens in better vaccines is clearly possible.

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Literature Cited

1. Aldorete, J. F., Robertson, D. C. 1978. *Infect. Immun.* 19:1021–30
2. Archer, G., Fekety, F. R. 1976. *J. Infect. Dis.* 134:1–7
3. Bacha, P., Murphy, J., Reichlin, S. 1983. *J. Biol. Chem.* 258:1565–70
4. Bagdasarian, M., Bagdasarian, M. M., Coleman, S., Timmis, K. N. 1979. In *Plasmids of Medical, Environmental, and Commercial Importance*, ed. K. N. Timmis, A. Puhler, pp. 411–22. New York: Elsevier/No. Holland
5. Barany, F., Boeke, J. D., Tomasz, A. 1982. *Proc. Natl. Acad. Sci. USA* 79: 2991–95
6. Bindereif, A., Neilands, J. B. 1983. *J. Bacteriol.* 153:1111–13
7. Binns, M. M., Davies, D. L., Hardy, K. G. 1979. *Nature* 279:778–81
8. Black, J. R., Black, W. J., Cannon, J. G. 1983. *Abstr. 23rd Intersci. Conf. Antimicrob. Ag. Chemother.* 138:D3. Washington DC: Am. Soc. Microbiol.
9. Black, W. J., Cannon, J. G. 1983. *Abstr: 83rd Ann. Meet. Am. Soc. Microbiol.* 60:D6. Washington DC: Am. Soc. Microbiol.
10. Blackwood, L. L., Stone, R. M., Iglewski, B. H., Pennington, J. E. 1983. *Infect. Immun.* 39:198–201

11. Bricker, J., Mulks, M. H., Plaut, A., Moxon, E. R., Wright, A. 1983. *Proc. Natl. Acad. Sci. USA* 80:2681–85
12. Broome, S., Gilbert, W. 1978. *Proc. Natl. Acad. Sci. USA* 75:2746–49
12a. Burdett, V., Beachey, E. 1984. *Abstr: 84th Ann. Meet. Am. Soc. Microbiol.* 25:B46. Washington DC: Am. Soc. Microbiol.
13. Burgess, N. M., Bywater, R. J., Cowley, C. M., Mullan, N. A., Newsome, P. M. 1978. *Infect. Immun.* 21:526–31
14. Cheng, S., Modrich, P. 1983. *J. Bacteriol.* 154:1005–8
15. Cheung, H., Schnepf, H. E., Whiteley, H. R. 1983. *J. Biol. Chem.* 258:1960–67
16. Clegg, S. 1982. *Infect. Immun.* 38:739–44
17. Clements, J. D., Yancey, R. J., Finkelstein, R. A. 1980. *Infect. Immun.* 29:91–97
18. Coleman, K., Dougan, G., Arbuthnott, J. P. 1983. *J. Bacteriol.* 153:909–15
19. Curtiss, R., Robeson, J. B., Barletta, R., Abiko, Y., Smorawinska, M. 1982. In *Microbiology 1982*, ed. D. Schlessinger, pp. 253–57. Washington DC: Amer. Soc. Microbiol.
20. Dallas, W. S. 1983. *Infect. Immun.* 40:647–52
21. Dallas, W. S., Falkow, S. 1979. *Nature* 277:406–7
22. Dallas, W. S., Falkow, S. 1980. *Nature* 288:499–501
23. Dallas, W. S., Gill, D. M., Falkow, S. 1979. *J. Bacteriol.* 139:850–58
24. Danner, D. B., Pifer, M. L. 1982. *Gene* 18:101–05
25. Dean, D. 1981. *Gene* 15:99–102
26. De La Cruz, F., Mullen, D., Ortiz, J., Goebel, W. 1980. *J. Bacteriol.* 143:825–33
27. De La Cruz, F., Zabala, J. C., Ortiz, J. M. 1983. *Infect. Immun.* 41:881–87
28. Ditta, G., Stanfield, S., Corbin, D., Helinski, D. R. 1980. *Proc. Natl. Acad. Sci. USA* 12:7347–51
29. Dougan, G., Dowd, G., Kehoe, M. 1983. *J. Bacteriol.* 153:364–70
30. Dubnau, D. 1983. In *Experimental Manipulation of Gene Expression*, ed. M. Inouye, in press. New York: Academic
31. Durack, D. T., Beeson, P. B. 1977. *Infect. Immun.* 16:213–17
32. Echarti, C., Hirschel, B., Boulnois, G., Varley, J., Waldvogel, F., Timmis, K. N. 1983. *Infect. Immun.* 41:54–60
33. Eisenstein, B. J. 1982. *Infection* 10:112–15
34. Elwell, L. P., Shipley, P. L. 1980. *Ann. Rev. Microbiol.* 34:465–96
35. Engleberg, N. C., Drutz, D. J., Eisenstein, B. J. 1983. *Abstr. 23rd Intersci. Conf. Antimicrob. Ag. Chemother.* 231:800. Washington DC: Am. Soc. Microbiol.
36. Fairweather, N., Kennedy, S., Foster, T. J., Kehoe, M., Dougan, D. 1983. *Infect. Immun.* 41:1112–17
37. Ferrari, F. A., Nguyen, A., Lang, D., Hoch, J. A. 1983. *J. Bacteriol.* 154: 1513–15
38. Field, M., Graf, L. H., Laird, W. J., Smith, P. L. 1978. *Proc. Natl. Acad. Sci. USA* 75:2800–4
39. Frazer, A. C., Curtiss, R. 1975. *Curr. Topics Microbiol. Immunol.* 69:1–84
40. Freedman, M. L., Tanzer, J. M. 1974. *Infect. Immun.* 10:189–96
41. Gaastra, W., Mooi, F. R., Stuitze, A. R., de Graaf, J. K. 1981. *FEMS Microbiol. Lett.* 12:41–46
42. Ganesan, A. T., Chang, S., Hoch, J. A. 1982. *Molecular Cloning and Gene Expression in Bacilli.* New York: Academic. 359 pp.
43. Ganesan, S., Kamdar, H., Jayaraman, K., Szulmajster, J. 1983. *Mol. Gen. Genet.* 189:181–83
44. Gennaro, M. L., Greenaway, P. J. 1983. *Nucl. Acids Res.* 12:3855–61
45. Gennaro, M. L., Greenaway, P. J., Broadbent, D. A. 1982. *Nucl. Acids Res.* 10:4883–90
46. Gill, D. M., Richardson, S. H. 1980. *J. Infect. Dis.* 141:64–70
47. Goebel, W., Hedgpeth, J. 1982. *J. Bacteriol.* 151:1290–98
48. Goebel, W., Schremph, H. 1971. *J. Bacteriol.* 106:311–17
49. Gonzalez, J. M., Brown, B. J., Carlton, B. C. 1982. *Proc. Natl. Acad. Sci. USA* 79:6951–55
50. Goze, A., Ehrlich, S. D. 1980. *Proc. Natl. Acad. Sci. USA* 77:7333–37
51. Gryczan, T. J., Dubnau, D. 1982. *Gene* 20:459–69
52. Gyles, C. L. 1979. *Can. J. Comp. Med.* 43:371–79
53. Hacker, J., Hughes, C., Hof, H., Goebel, W. 1983. *Infect. Immun.* 42:57–63
54. Haldenwang, W. G., Banner, C. D. B., Ollington, J. F., Losick, R., Hoch, J. A., et al. 1980. *J. Bacteriol.* 142:90–95
55. Hansen, J. B., Abiko, Y., Curtiss, R. 1981. *Infect. Immun.* 31:1034–43
56. Held, G. A., Bulla, L. A., Ferrari, E., Hoch, J., Aronson, A. J., et al. *Proc. Natl. Acad. Sci. USA* 79:6065–69
57. Holt, R. G., Abiko, Y., Saito, S., Smorawinska, M., Hansen, J., et al. 1982. *Infect. Immun.* 38:147–56
58. Hull, R. A., Gill, R., Hsu, P., Minshew, B., Falkow, S. 1981. *Infect. Immun.* 33:933–38

59. Hughes, C., Hacker, J., Roberts, A., Goebel, W. 1983. *Infect. Immun.* 39: 546–51
60. Hughes, M., Machardy, S. M., Sheppard, A. J., Woods, N. C. 1980. *Infect. Immun.* 27:576–88
61. Hughes, J. M., Murad, F., Chang, B., Guerrant, R. L. 1978. *Nature* 271:755–56
62. Isaacson, R. E. 1983. *Abstr. 83rd Ann. Meet. Am. Soc. Microbiol.* D8:60. Washington DC: Am. Soc. Microbiol.
63. Jagusztyn-Krynicka, E. K., Smorawinska, M., Curtiss, R. 1982. *J. Gen. Microbiol.* 128:1135–45
64. Kallenius, G., Mollby, R., Winberg, J. 1980. *Infect. Immun.* 28:972–80
65. Kaper, J. B., Levine, M. M. 1981. *Lancet*, Vol. II, Nov. 21, 1981. pp. 1162–63
66. Kehoe, M., Duncan, J., Foster, T., Fairweather, N., Dougan, G. 1983. *Infect. Immun.* 41:1105–11
67. Kehoe, M., Sellwood, R., Shipley, P., Dougan, G. 1981. *Nature* 291:122–26
68. Kehoe, M., Winther, M., Dougan, G. 1983. *J. Bacteriol.* 155:1071–77
69. Keller, G., Schleifer, K., Gotz, F. 1983. *Plasmid* 10:270–78
70. Klier, A., Bourgouin, C., Rapoport, G. 1983. *Mol. Gen. Genet.* 191:257–62
71. Klier, A., Fargette, F., Ribier, J., Rapoport, G. 1982. *EMBO J.* 1:791–99
72. Klier, A., Parsot, C., Rapoport, G. 1983. *Nucl. Acids Res.* 11:3973–87
72a. Koomey, J. M., Falkow, S. 1984. *Infect. Immun.* 43:101–7
73. Koomey, J. M., Gill, R. E., Falkow, S. 1982. *Proc. Natl. Acad. Sci. USA* 79: 7881–55
74. Koomey, J. M., Welch, R. A., Falkow, S. 1982. In *Antibiotics in the Management of Infections: Outlook for the 1980s*, ed. A. G. Bearn, pp. 31–45. New York: Raven
75. Kopecko, D. J., Schneider, H., Seid, R., Zollinger, W., Wohlhieter, J. A. 1983. *Abstr. 83rd Ann. Meet. Am. Soc. Microbiol.* 60:D9. Washington DC: Am. Soc. Microbiol.
76. Kreft, J., Berger, H., Hartlein, M., Muller, B., Weidinger, G., Goebel, W. 1983. *J. Bacteriol.* 155:681–89
77. Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., Novick, R. P. 1983. *Nature* 305:709–12
78. Kronstad, J. W., Schnepf, H. E., Whiteley, H. R. 1983. *J. Bacteriol.* 154: 419–28
79. Kuramitsu, H. K., Wondrack, L. 1983. *Infect. Immun.* 42:763–70
80. LeBlanc, D. J., Lee, L. N., Clewell, D. B., Behnke, D. 1983. *Infect. Immun.* 40:1015–22
81. Lee, C. H., Moseley, S. L., Moon, H. W., Whipp, S. C., Gyles, C. L., So, M. 1983. *Infect. Immun.* 42:264–68
82. Leong, D., Coleman, K. O., Murphy, J. R. 1983. *Science* 220:515–17
83. Lingood, M. A., Ingram, P. L. 1982. *J. Med. Microbiol.* 15:23–30
84. Liu, P. V. 1974. *J. Infect. Dis.* 130:S94–S99
85. Lofdahl, S., Guss, B., Uhlen, M., Philipson, L., Lindberg, M. 1983. *Proc. Natl. Acad. Sci. USA* 80:697–71
86. Lory, S., Tai, P. C. 1983. *Gene* 22:95–101
87. Macrina, F. L., Ash Tobian, J., Evans, R. P., Jones, K. R. 1982. In *Microbiology 1982*, ed. D. Schlessinger, pp. 234–38. Washington DC: Am. Soc. Microbiol.
88. Macrina, F. L., Ash Tobian, J. Jones, K. R. Evans, R. P., Clewell, D. B. 1982. *Gene* 19:345–53
89. Macrina, F. L., Evans, R. P., Ash Tobian, J. A., Hartley, D. L., Clewell, D. B., et al. 1983. *Gene* 25: in press
89a. Malke, H., Ferretti, J. 1984. *Proc. Natl. Acad. Sci. USA* In press
90. Maniatis, T., Fritsch, E. F., Sambrook, J. 1982. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spr. Harb. Lab.
91. Maxam, A. M., Gilbert, W. 1980. *Methods in Enzymology* 65:499–560
92. McCarthy, D., Clayton, N. L., Setlow, J. K. 1982. *J. Bacteriol.* 151: 1605–7
93. Meyer, T. F., Mlawer, N., So, M., 1982. *Cell* 30:45–52
94. Moll, A., Manning, P. A., Timmis, K. N. 1980. *Infect. Immun.* 28:359–67
95. Mooi, F. R., de Graaf, F. K., van Embden, J. O. A. 1979. *Nucl. Acids Res.* 6:849–65
96. Mooi, F. R., Harms, N., Bakker, D., de Graaf, F. K. 1981. *Infect. Immun.* 32:1155–63
97. Moseley, S. L., Echeverria, P., Seriwatana, C., Tirapat, C., Chaicumpa, W., et al. 1982. *J. Infect. Dis.* 145:863–69
98. Moseley, S. L., Falkow, S. 1980. *J. Bacteriol.* 144:444–46
99. Moseley, S. L., Hardy, J. W., Huq, M. I., Echeverria, P., Falkow, S. 1983. *Infect. Immun.* 39:1167–74
100. Moseley, S. L., Samadpour-Motalebi, M., Falkow, S. 1983. *J. Bacteriol.* 156: 441–43
101. Muller, D., Hughes, C., Goebel, W. 1983. *J. Bacteriol.* 153:846–51
102. Neidhardt, F. C., Vaughn, V., Phillips,

T. A., Block, P. L. 1983. *Microbiol. Rev.* 47:231–84
103. Neidhardt, F. C., Wirth, R., Smith, M. W., Van Bogelen, R. 1980. *J. Bacteriol.* 143:535–37
104. Noegel, A., Rdest, U., Goebel, W. 1981. *J. Bacteriol.* 145:233–47
105. Noegel, A., Rdest, U., Springer, W., Goebel, W. 1979. *Mol. Gen. Genet.* 175:343–50
106. Norgard, M. V., Miller, J. N. 1983. *Infect. Immun.* 42:435–45
107. Normark, S., Lark, D., Hull, R., Norgren, M., Baga, M., et al. 1983. *Infect. Immun.* 41:942–49
108. Olsen, R. H., DeBusscher, G., McCombie, W. R. 1982. *J. Bacteriol.* 150:60–69
109. Pappenheimer, A. M. 1977. *Ann. Rev. Biochem.* 46:69–94
110. Pearson, G. D. N., Mekalanos, J. J. 1982. *Proc. Natl. Acad. Sci. USA* 79: 2976–80
111. Picken, R. N., Mazaitis, A. J., Maas, W. K., Rey, M., Heymeker, H. 1983. *Infect. Immun.* 42:269–75
112. Plaut, A. 1983. *Ann. Rev. Microbiol.* 37:603–22
112a. Poirier, T., Kehoe, M., Dale, J., Timmis, K., Beachey, E. 1984. *Abstr. 84th Ann. Meet. Am. Soc. Microbiol.* 25:B45. Washington DC: Am. Soc. Microbiol.
113. Purcell, B. K., Clegg, S. 1983. *Infect. Immun.* 39:1122–27
114. Ratti, G. Rappuoli, R., Giannini, G. 1983. *Nucl. Acids Res.* 11:6589–95
115. Reeve, J. 1979. *Methods in Enzymol.* 68:493–503
116. Rhen, M., Knowles, J., Penttila, M. E., Sarvas, M., Korhonen, T. K. 1983. *FEMS Microbiol. Lett.* 19:119–23
117. Rhen, M., Makela, P. H., Korhonen, T. K. *FEMS Microbiol. Lett.* 19:267–71
118. Roberts, T. M., Swanberg, S. L., Poteete, A., Riedel, G., Blackman, K. 1980. *Gene* 12:123–27
119. Robeson, J. P., Barletta, R. G., Curtiss, R. 1983. *J. Bacteriol.* 153:211–21
120. Ruvkun, G. B., Ausubel, F. M. 1981. *Nature* 289:85–88
121. Sako, T., Sawaki, S., Sakurai, T., Ito, S., Yoshizawa, Y., et al. *Mol. Gen. Genet.* 190:271–77
122. Sancar, A., Hack, A. M., Rupp, W. D. 1979. *J. Bacteriol.* 137:692–93
123. Sanger, F., Nicklen, S., Coulson, A. R. 1977. *Proc. Natl. Acad. Sci. USA* 74:5463–67
124. Schiffer, M. S., Oliveira, E., Glode, M. P., McCracken, G. H., Sarff, M., et al. 1976. *Pediatr. Res.* 10:82–89
125. Schnepf, H. E., Whiteley, H. R. 1981. *Proc. Natl. Acad. Sci. USA* 78:2893–97
126. Schumann, W. 1979. *Mol. Gen. Genet.* 174:221–24
127. Scott, J. R., Fischetti, V. A. 1983. *Science* 221:758–60
128. Shipley, P., Dougan, G., Falkow, S. 1981. *J. Bacteriol.* 145:920–25
129. Shortle, D., DiMaio, D., Nathans, D. 1981. *Ann. Rev. Genetics* 15:265–94
130. Silver, R. P., Finn, C. W., Vann, W. F., Aaronson, W., Schneerson, R., et al. 1981. *Nature* 289:696–98
131. Silverman, M., Simon, M. 1980. *Cell* 19:845–54
132. Silverman, M., Zieg, J., Simon, M. 1979. *J. Bacteriol.* 137:517–23
133. Simon, M., Zieg, J., Silverman, M., Mandel, G., Doolittle, R. 1980. *Science* 209:1370–74
134. Skalka, A., Shapiro, L. 1976. *Gene* 1: 65–79
135. Smith, H. W., Halls, S. 1967. *J. Gen. Microbiol.* 47:153–61
136. Smith, H. W., Linggood, M. A. 1971. *J. Med. Microbiol.* 4:467–85
137. Smorawinska, M., Hsu, J. C., Hansen, J. B., Jagusztyn-Krynicka, E. K., Abiko, Y., et al. 1983. *J. Bacteriol.* 153: 1095–97
138. So, M., Atchison, S., Falkow, S., Moseley, S., McCarthy, B. J. 1980. *Cold Spr. Harb. Symp. Quant. Biol.* 45:53–58
139. So, M., Boyer, H. W., Betlach, M., Falkow, S. 1976. *J. Bacteriol.* 128:463–72
140. So, M., Dallas, W. S., Falkow, S. 1978. *Infect. Immun.* 21:405–11
141. So, M., Heffron, F., McCarthy, B. J. 1979. *Nature* 277:553–56
142. So, M., McCarthy, B. J. 1980. *Proc. Natl. Acad. Sci. USA* 77:4011–15
143. Sparling, P. F. 1979. In *Microbiology 1979*, ed. D. Schlessinger, pp. 249–53. Washington DC: Amer. Soc. Microbiol.
144. Spicer, E. K., Kavanaugh, W. M., Dallas, W. S., Falkow, S., Konigsberg, W. H., et al. 1981. *Proc. Natl. Acad. Sci. USA* 78:50–54
145. Stamm, L. V., Bassford, P. J. 1982. *DNA* 1:329–32
146. Stamm, L. V., Folds, J. D., Bassford, P. J. 1982. *Infect. Immun.* 36:1238–41
147. Stamm, L. V., Kerner, T. C., Bankaitis, V. A., Bassford, P. J. 1983. *Infect. Immun.* 41:709–21
148. Stark, J. M., Shuster, C. W. 1983. *Plasmid* 10:45–54
149. Stassi, D., Lopez, P., Espinosa, M., Lacks, S. A. 1981. *Proc. Natl. Acad. Sci. USA* 78:7028–32
150. Stein, D. C., Young, F. E., Tenover, F. C., Clark, V. L. 1983. *Mol. Gen. Genet.* 189:77–84

151. Stephens, R. S., Kuo, C., Agabian, N. 1983. *Abstr. 83rd Ann. Meet. Am. Soc. Microbiol.* 35:B69. Washington DC: Am. Soc. Microbiol.
152. Swanson, J. 1973. *J. Exp. Med.* 127:571–89
153. Szekely, E., Simon, M. 1983. *J. Bacteriol.* 155:74–81
154. Tanzer, J. M., Freedman, M. L., Fitzgerald, R. J., Larson, R. H. 1974. *Infect. Immun.* 10:197–203
155. Taylor, M. A., McIntosh, M. A., Robbins, J., Wise, K. S. 1983. *Proc. Natl. Acad. Sci. USA* 80:4154–58
156. Tweten, R. K., Collier, R. J. 1983. *J. Bacteriol.* 156:680–85
157. van den Bosch, J. F., Verboom-Sohmer, U., Postma, P., de Graaf, J. MacLaren, D. M. 1980. *Infect. Immun.* 29:226–33
158. van Die, I., van der Hondel, C., Hamstra, H., Hoekstra, W., Bergmans, H. 1983. *FEMS Microbiol. Lett.* 19:77–82
159. van Embden, J. D. A., de Graaf, F. K., Schouls, L. M., Teppema, J. S. 1980. *Infect. Immun.* 29:1125–33
160. van Embden, J. D., van der Donk, R. V., van Eijk, H. G., van der Heide, J., de Jong, J. A., et al. 1983. *Infect. Immun.* 42:187–96
161. Vasil, M. L., Berka, R. M., Gray, G. L., Nakai, H. 1982. *J. Bacteriol.* 152:431–40
162. Vasseghi, H., Claverys, J. P., Sicard, A. M. 1981. In *Transformation 1980*, ed. M. Polsinelli, G. Mazza, pp. 137–54. Oxford, UK: Cotswold
163. Vodkin, M. H., Leppla, S. H. 1983. *Cell* 34:693–97
164. Waalwijk, C., van den Bosch, J. F., MacLaren, D. M., de Graaf, F. K. 1982. *Infect. Immun.* 35:32–37
165. Wagner, W., Vogel, M., Goebel, W. 1983. *J. Bacteriol.* 154:200–10
166. Welch, R. A., Dellinger, E. P., Minshew, B., Falkow, S. 1981. *Nature* 294:665–67
167. Welch, R. A., Falkow, S. 1984. *Infect. Immun.* 43:156–60
168. Welch, R. A., Hull, R., Falkow, S. 1983. *Infect. Immun.* 42:178–86
169. Wenman, W. M., Lovett, M. A. 1982. *Nature* 296:68–70
170. Williams, P. H., Warner, P. J. 1980. *Infect. Immun.* 29:411–16
171. Willshaw, G. A., Smith, H. R., Rowe, B. 1983. *FEMS Microbiol. Lett.* 16: 101–6
172. Winter, R. B., Gold, L. 1983. *Cell* 33:877–85
173. Wood, D. O., Atkinson, W. H., Sikorshi, R. S., Winkler, H. H. 1983. *J. Bacteriol.* 155:412–16
174. Wood, D. O., Hollinger, M. F., Tindol, M. B. 1981. *J. Bacteriol.* 14:1448–51
175. Wu, R., ed. 1979. *Methods in Enzymology*, vol. 68. New York: Academic
176. Yamamoto, T., Tamura, T., Ryoji, M., Kaji, A., Yokota, T., 1982. *J. Bacteriol.* 152:506–9
177. Yamamoto, T., Tamura, T., Yokota, T., Takano, T. 1982. *Mol. Gen. Genet.* 188:356–59
178. Yamamoto, T., Yokota, T. 1980. *J. Bacteriol.* 143:652–60
179. Yamamoto, T., Yokota, T. 1981. *J. Bacteriol.* 145:850–60
180. Yamamoto, T., Yokota, T. 1983. *J. Bacteriol.* 153:1352–60
181. Yamamoto, T., Yokota, T. 1983. *J. Bacteriol.* 155:728–33
182. Yamamoto, T., Yokota, T., Kaji, A. 1981. *J. Bacteriol.* 148:983–87
183. Young, R. A., Davis, R. W. 1983. *Science* 222:778–82
184. Zieg, J., Silverman, M., Hilmen, M., Simon, M. 1977. *Science* 196:170–72
185. Zieg, J., Simon, M. 1980. *Proc. Natl. Acad. Sci. USA* 77:4196–260

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SYNTHETIC VIRAL VACCINES

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INTRODUCTION

Vaccines in the Control of Virus Diseases

Vaccination has been the main factor in the control of and—in the case of smallpox—eradication of virus diseases during the last 30 years. There can be no doubting the extraordinary success of vaccines in controlling yellow fever, poliomyelitis, small pox, and German measles in the human population, and

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equally effective vaccines against Newcastle disease and foot-and-mouth disease have been used in domestic animals.

There are two different kinds of vaccine in general use. In the first, the so-called attenuated vaccines, virulent strains of the viruses are grown in either an "unnatural" host or in a host cell until they no longer produce disease in the natural host but grow sufficiently well to invoke an immune response. There are numerous examples of this method for producing a vaccine. The two best known probably are the yellow fever 17D strain developed by passage in mice and then in chick embryos (63) and the poliomyelitis strains developed by passaging in monkey kidney cells (54). Despite the lack of a known scientific basis, this procedure has provided several outstandingly successful products.

Variations on this general theme, such as passaging the virus at a temperature lower than normal (cold-adapted mutants), have also been used and some promise is shown by Maassab's influenza virus strain (35).

Naturally occurring attenuated viruses have also been highly successful. The best known example is of course the use of cowpox virus to prevent smallpox. Indeed the use of cowpox virus for this purpose heralded the practice of vaccination for the control of a virus disease, although the history of the strains actually used by Jesty & Jenner is obscure. Another example is provided by the highly successful use of turkey herpes virus to control Marek's disease of chickens.

In the second type of vaccine, the virus is grown in large amounts, usually in tissue-culture cells, and then inactivated with an agent such as formaldehyde, β-propiolactone, or, more recently, an imine. With these so-called killed vaccines, a sufficient mass of virus particles is required to invoke the antibody response required to protect the recipient.

The response to the two types of vaccine is different. With the attenuated viruses the aim is to produce infection and thus reproduce the complete range of immune responses obtained in natural infection, but without causing disease. With killed vaccines the response is generally regarded as being the production of protective antibody. Clearly attenuated vaccines are preferable on the grounds of the range of immune responses that is elicited.

Problems with Currently Available Vaccines

Both types of vaccine present problems in their production and quality control. In the first place, it is vital to preserve the correct level of attenuation in living vaccines. This is done in practice by use of a seed-lot system so that the virus used in the manufacture of the vaccine is at the correct passage level. Passage generally reduces virulence, but there are instances where increase in virulence has been observed following tissue-culture passage. Another hazard in the use of attenuated vaccines is the possibility of contamination with other viruses due to their adventitious presence in the cells or medium used for production.

The shelf life of attenuated vaccines is limited by the stability of the virus at the temperature of storage. Where vaccines have to be applied in tropical climates this problem can assume considerable importance.

Even with highly successful products such as the attenuated poliovirus and smallpox vaccines, there is a small but significant number of postvaccination incidents. The eradication of smallpox has clearly solved the problem with this vaccine, but there is a sufficient number of poliomyelitis cases each year attributable to the vaccine to warrant efforts to establish the scientific basis for attenuation with this virus and to apply the rationale to the development of a safer vaccine.

Killed vaccines also present problems in their manufacture. Thus there is the constant problem of ensuring that the product is innocuous. It is impossible to provide a practical solution to this problem, because the only certain method would be to test all the vaccine. Fortunately, formaldehyde is now used as an inactivant to a lesser extent than previously, so the chance of infectious virus surviving the inactivation process is correspondingly reduced.

Storage of inactivated vaccines, as with attenuated strains, can also present a problem. Thus foot-and-mouth disease virus vaccines, which depend for their efficacy on the maintenance of the integrity of the virus particle, have a limited shelf life, even at 4°C, because of the vulnerability of the antigenic determinant to proteolytic enzymes. Another problem that cannot be disregarded is the hazard, both to the personnel involved and to the environment, of working with highly virulent agents.

Need for New Approaches

The above problems associated with the production of virus vaccines warrant the search for better or more dependable products. Thus it seems desirable that the empirical way in which attenuated vaccine strains have been selected should be replaced by a more rational approach, in which account is taken of the chemical basis for attenuation. Already the comparisons of the nucleotide sequences of the neurovirulent and corresponding attenuated strains of poliovirus that have been made (58) are throwing light on this problem.

With killed vaccines, it is clearly necessary to recognise the protein carrying the immunizing antigen. This has been done with several viruses (see next section), and with the development of techniques for cloning and sequencing nucleic acids coding for virus proteins, it is now possible to identify antigenic regions on a few viruses. This has led to the organic and biological synthesis of peptides corresponding to these regions.

VIRUS SUBUNITS AS VACCINES

Electron Microscopy

For more than 20 years, virologists interested in vaccines have sought to identify those parts of a virus that are important in immunization. The visualization of viruses in the electron microscope was a major step in these studies. The general application of the negative-staining technique, introduced in 1959 (11), revealed the enormous diversity of virus structure. With the parallel development of techniques for the purification of macromolecules, it has become possible to obtain many viruses in a highly purified form and to analyze their constituents. By combining with these methods the use of radioactively labeled precursor molecules for nucleic acids, proteins, lipids, and carbohydrates, the structure of many viruses has been revealed in fine detail. This work has confirmed the enormous diversity in structure that had been foreshadowed by electron microscopy studies. This diversity is summarized diagrammatically by Matthews (38).

Dissection of Virus Particles into Biologically Active Entities

From the observation that many viruses lost infectivity when treated with lipid solvents (4) came the realization that these viruses contained a lipid envelope. Indeed, Almeida & Waterson (1) used this property as an important parameter for virus classification. Of more importance for the purpose of elucidating structure, however, was the observation that the loss of infectivity was frequently accompanied by the release of fragments with a characteristic morphology and, significantly, biological activity. This approach has allowed the protein(s) associated with immunizing activity to be recognized. One of the best examples from the point of view of morphology is provided by vesicular stomatitis virus, a rhabdovirus that is closely related structurally to rabies virus.

These viruses contain five proteins. It was found that treatment of vesicular stomatitis virus with ether or a mild detergent resulted in the loss of more than 99.99% of the original infectivity (12). Examination in the electron microscope revealed the presence of spring-like particles devoid of lipid and the surface spike projections. Fractionation of the products in sucrose gradients showed that the residual infectivity was associated with these particles, but the capacity to elicit neutralizing antibody was associated with a fraction of 70,000 daltons. Subsequent polyacrylamide gel electrophoresis showed that the immunogenic fraction contained only one protein, the other four being associated with the spring-like particles. The association of the immunizing activity with the surface projections was confirmed by the observation that trypsin removed them from the surface of the virus, leaving a particle that had lost the capacity to elicit neutralizing antibody.

Similar results have been obtained with several viruses possessing lipid envelopes. The surface projections from the viruses of, for example, influenza, measles, rabies, and Semliki Forest (14, 15, 23, 43) have all been shown to be active in eliciting neutralizing antibody. In most instances, however, the released protein has much lower activity than the intact virion. However, by incorporating the subunits into micellar structures (43, 44, 56), this activity can be enhanced until it approaches that of the virus particle. Clearly the conformation of the released protein is different from the one it assumes on the virus particle, and incorporation into micelles restores the protein to this original conformation.

It has also been found possible to disrupt viruses not possessing a lipid envelope (the so-called naked viruses) into biologically active fragments, although more vigorous conditions are required. The best known example is foot-and-mouth disease virus. It was shown several years ago (66) that incubation of the virus with trypsin resulted in the loss of infectivity and immunizing activity. The trypsin-treated particles differed from the untreated particles in that one of the four capsid proteins, namely VP1, had been cleaved. Subsequent work by several groups of workers (5, 29, 33, 40) has been shown that the isolated protein elicits neutralizing antibody in several species of animals. Although the activity of the released protein is considerably lower than that of the intact virus particle, two injections of ~ 500 μg are sufficient to protect pigs and cattle against challenge infection.

Advantages of Defined Antigens as Vaccines

These observations that released proteins possess significant, albeit low, immunizing activity paved the way to considerations of their potential value as vaccines. The advantages of using isolated proteins as immunogens would be: (*a*) their stability; (*b*) their freedom from extraneous proteins and nucleic acids, and (*c*) their precise composition.

There is, however, the considerable disadvantage, referred to above, that the isolated protein has a much lower activity compared with that of the intact virus particle. This lower activity is most likely due to the different configuration that the separated protein takes up when it is released from the constraints imposed by the architecture of the virus particle.

An interesting example is provided by the low activity of the isolated 12S protein subunit of foot-and-mouth disease virus. The intact virus particle consists of one molecule of ssRNA, M_r ~ 2,600,000, and 60 copies of each 4 proteins VP1–4. The M_r of VP1-VP3 is ~ 24,000, and that of VP4 is ~ 8,000. It is known that the major immunogenic site is located on VP1. However, the 12S protein subunit produced by gentle disruption (pH 6.5) of the virus particle has only about 1% of the activity of the virus particle, although it contains VP1 in an undegraded form as part of a molecule of 350,000 daltons. (The 12S protein is a pentamer of VP1, VP2, and VP3).

BIOSYNTHESIS OF ANTIGENIC PROTEINS

Several methods have been worked out for expressing proteins. These can be divided into those in which the protein is expressed in in vitro cultures of prokaryotic or eukaryotic cells and those in which the DNA sequence coding for the desired product is inserted into a virus that is then allowed to replicate in vitro or in vivo. The recent description by Moss (36), Paoletti (48), and their colleagues of the use of vaccinia virus for the in vivo expression of foreign genes is probably only the forerunner of other viruses that will be used for this purpose.

Expression in Bacterial Cells

The first expression experiments were done using *E. coli* cells. The hemagglutinin of fowl plague (influenza) virus, which is located on the surface of the virus particle, is responsible for cell attachment and the production of neutralizing antibody. The RNA of influenza virus consists of eight segments of single-stranded RNA, and the segment coding for the HA has been identified. The complete HA protein was expressed in *E. coli* cells by cloning a cDNA copy of this segment into an expression vector downstream from a trp promoter (20). Although protein reacting with antivirus serum was detected in the induced bacteria, the yields were very low. In more recent work, higher yields were obtained and the product elicited antibodies that recognized native HA (16). Unfortunately no protective studies have been done with these expressed products.

Other virus glycoproteins have been expressed by essentially the same method. Those include the immunizing proteins of the viruses causing rabies, vesicular stomatitis, herpes, and hepatitis (22, 52, 65, 69). In some instances low levels of expression were obtained unless the DNA coding for the hydrophobic region of the protein was removed.

By far the greatest success was obtained with the immunizing protein VP1 of foot-and-mouth disease virus. The position of this protein on the virus genome had been determined previously by biochemical mapping experiments (55), and the cDNA clones coding for this region had been obtained by reverse transcription of the virus RNA (9, 31). Successful expression was obtained by Kleid et al (30) by making use of a vector that was designed to produce a hybrid protein containing a trpLE fragment that is insoluble and resistant to proteolysis. Because of this property as much as 17% of the total cell protein could be obtained as the hybrid protein. This protein elicited neutralizing antibodies in experimental animals and in the naturally susceptible pigs and cattle. After two or three injections the pigs and cattle withstood challenge with the homologous virus. However, the amount required for successful immunization was much greater than the weight of inactivated virus particles. Clearly the configuration

of VP1 in the expressed protein differs from that it assumes in the intact virus particle. The pressing need is to determine the configuration of VP1 on the virus particle and to ascertain the factors that will allow the expressed protein to fold in the same way.

Expression in Eukaryotic Cells

One of the limitations of *E. coli* expression is that the products are not glycosylated. Because of this, other organisms have been studied in which it is known that glycosylation takes place. Yeast cells have been used for the expression of the surface protein of hepatitis B virus (27, 47, 64). This virus causes a debilitating disease, and many millions of cases are reported each year. The only vaccine currently in use is based on the 22 nm glycoprotein particles found in the blood of asymptomatic carriers of the disease. These particles are purified extensively, to ensure that there is no residual infectious virus in the product.

Clearly this method of production is expensive and leaves unanswered the awkward question of whether other adventitious agents such as that causing acquired immune deficiency syndrome (AIDS) have been rendered innocuous by the purification procedure. Safety testing, which is done in chimpanzees, is expensive, and the arguments about the number of animals required to ensure the innocuity of the product are well known.

Valenzuela et al (64) reported in 1982 that yeast cells are able not only to express the surface antigen gene but also to assemble the polypeptide into particles that have the same morphology as those isolated from human plasma. The product was immunogenic in mice. Other groups (27, 47) have since reported that the yeast product is antigenic in rabbits and guinea pigs. In the most recent development of this work (39), the product has been shown to elicit high neutralizing antibody levels in mice, grivet monkeys, and chimpanzees and, more significantly, to protect the vaccinated chimpanzees against intravenous challenge with the virus. The vaccine was as potent as that derived from human plasma and the antibody produced by the yeast product persisted for at least a year in monkeys.

The same group of workers (39) has also shown that the vaccine affords protection against a heterologous subtype of the virus. This result indicates that the dominant "a" antigen is also dominant in the yeast product. These results are a landmark in recombinant DNA technology because they provide the first example of a vaccine that is effective against a human virus infection.

Because most of the molecules of the surface antigen expressed in yeast are either not glycosylated or inefficiently glycosylated, several attempts have been made to express it in higher eukaryotic cells where it is expected that complete glycosylation should occur. A human hepatoma cell line (6), simian virus 40–infected monkey kidney cells (45), and mouse L cells (18) have all

been used. Clearly several options are now open for the production of a hepatitis B vaccine, but it is questionable whether the use of these higher eukaryotic cells would be acceptable for a human vaccine because of their possible neoplastic nature.

Influenza virus hemagglutinin has also been expressed in monkey kidney cells infected with simian virus 40 in which either early or late genes had been replaced with a cloned copy of the hemagglutinin gene. Cells infected with these vectors produce appreciable amounts of the hemagglutinin in a glycosylated form (25, 26). Similar studies have also been carried out by Sveda and his colleagues (61, 62). These experiments illustrate the methods that are possible for the construction of vectors containing glycoproteins and their insertion into eukaryotic cells.

Expression in Virus Vectors

The principles governing the manipulation of eukaryotic viral genomes for the construction of vectors are similar to those that have been used for the development of the bacteriophage lambda vectors (10). The vector genome must be easily manipulated by having convenient sites for the restriction enzymes used in cloning, and the location of these sites with respect to vector control systems must be understood in detail.

Because animal viruses are subjected to rigid packaging contraints, there is a limit to the size of gene that can be introduced into the virus DNA. However, the large size of the adeno, herpes, and vaccinia virus genomes makes them extremely useful as vectors. The recent work by Moss (36) and Paoletti (48) and their colleagues has shown that it is possible to construct recombinant vaccinia virus genomes containing the herpes simplex virus thymidine kinase gene. Because vaccinia virus DNA is not infectious, it is necessary to perform the final stage of construction by in vivo recombination. This was done by inserting the plasmid containing the thymidine kinase gene into vaccinia virus–infected cells. The resultant vaccinia virus expressed the inserted gene when it replicated in the cells. Subsequently the same two groups have described the use of this approach for the expression of hepatitis B surface antigen (57) and other virus proteins.

CHEMICALLY DEFINED ANTIGENS

Proteins may carry a large number of sites that determine their antigenic specificity. However, it is now apparent that only a limited number of these sites are involved in evoking an immune response. These observations have provided the basis for the possible application of chemically defined immunogens as vaccines against virus diseases.

The major problem is the identification of the immunogenic sites on the virus protein. Predictions have been made by (*a*) measuring the biological activity of peptide fragments; (*b*) identifying the sites that differ in antigenic variants; (*c*) identifying the sites that react with neutralizing monoclonal antibodies; and (*d*) studying the secondary structure of the immunizing protein.

Predictions from the Biological Activity of Peptide Fragments

The first observation by this method was made by Anderer (2), who showed that short fragments of the protein of tobacco mosaic virus would inhibit the precipitation of the virus by its antiserum. All the fragments were at the COOH end of the protein, and further work by Anderer (3) showed that the hexapeptide from the C terminus, when coupled to bovine serum albumin, elicited the formation of antibodies that not only precipitated the intact virus but also neutralized its infectivity. Although this work was done with a digest of the native protein and was not in the true sense a synthetic product, it pointed the way to the possible use of short synthetic peptides as immunogens in the control of virus diseases.

A decade later Sela and his colleagues (32) showed that a peptide consisting of 21 residues of the 129 amino acid coat protein of MS2 bacteriophage elicited the production of antibody that linked to the virus particle. Moreover, this peptide inhibited the neutralization of the phage. The peptide was chemically synthesized and shown to elicit antibodies that attached to the virus particle. The further addition of antispecies antibody to the virus-antipeptide antiserum complex neutralized the virus. Although these observations did not demonstrate directly that antipeptide antiserum produced by injecting a synthetic peptide will neutralize the virus, the results were sufficiently encouraging to underline the potential of the synthetic vaccine approach.

The fact that these observations were not immediately followed up by other workers was due to the unavailability of the appropriate amino acid sequences. The introduction of cloning and nucleic acid sequencing techniques during the last decade has produced an avalanche of protein sequences. With the recognition that the immunogenic site(s) of viruses are usually found on a single protein and the biochemical mapping of virus genomes that has taken place during the same period, the way is clear for considerable advances.

A recent very good example is provided by the work with foot-and-mouth disease virus of Strohmaier and his colleagues (59). It had been recognized earlier (66) that the major immunogenic activity of the virus was associated with only one of the four capsid proteins. The German workers showed that two of the four fragments of the protein obtained by cyanogen bromide cleavage would elicit neutralizing antibody in mice. In addition they found that cleavage of the protein in situ by proteolytic enzymes also gave rise to fragments that

were immunogenic. The positions of the fragments on the VP1 molecule were established by partial sequencing at their N and C termini and comparing these sequences with that of the complete VP1 molecule deduced from the nucleic acid coding for it. By comparing the biological activities of the fragments of differing lengths from the same region of VP1, Strohmaier et al (59) predicted that tracts 146–154 and 200–213 of the 213 amino acid long protein would form part of immunogenic regions.

It has now been shown that organically synthesized peptides 141–160 (8) and 144–159 (49), each of which contains the predicted 146–154, elicit the formation of neutralizing antibody, but 146–154 itself did not elicit neutralizing antibody. The second predicted active tract elicited neutralizing antibody but to a much lesser extent than 141–160.

Predictions from Antigenic Variation

Although this approach is only applicable with viruses that show antigenic variability, the observations made recently with foot-and-mouth disease virus (FMDV) provide an example of the way in which it has been used to predict immunogenic sites. The virus exists as seven serotypes—A, O, C, SAT 1, SAT 2, SAT 3, and Asia 1—which are so different that infection with one serotype confers immunity only against homotypic challenge infection. Moreover, the variation within serotypes is such that immunization with one strain within a serotype may not necessarily confer protection against other strains within the same serotype. This clearly presents great practical difficulties in the control of disease but at the same time it provides the opportunity to study the chemical basis for antigenic variation.

It was shown several years ago by tryptic peptide mapping of the virus-induced proteins of three FMDV strains, two belonging to different subtypes of serotype A and the third to serotype O, that major differences were present in the capsid proteins VP1 and VP3, whereas the remainder were highly conserved (51). This observation was supported by Marquardt's work on the RNAs of different serotypes (37). He showed that homology between serotypes was ~ 20% in the capsid protein region, whereas it was as high as 80% in the non–capsid protein region.

Subsequent sequence studies of the RNAs of several viruses have revealed that VP1 is largely conserved but contains three regions of high variability, at 41–60, 134–160, and 195–213. The 41–60 region is largely hydrophobic and was considered to be internal. This in fact has been confirmed by the observation that antibody to this peptide does not attach to virus particles (53). Peptides corresponding to the other two variable regions elicited neutralizing antibody, with the 134–160 region being far more active.

Tracts of differing lengths in the 131–160 region have been tested for their activity in experimental animals and in cattle and pigs. These peptides have

been synthesised either chemically or in *E. coli* using cloned fragments of DNA (42) or, in one experiment, a chemically synthesized DNA corresponding to amino acid residues 142–160 (67). All the peptides produced good levels of neutralizing antibody, and in one experiment cattle given one injection of as little as 25 μg as part of a fusion protein were protected from in-contact challenge infection (J. J. Callis, unpublished observations). These highly encouraging results lead the author to the conclusion that peptide vaccines are commercially feasible even where, as with foot-and-mouth disease, the price of the current conventional vaccines is extremely low.

Application of the same approach to poliovirus showed that there were amino acid differences in the VP1 polypeptides of serotypes 1 and 3 at certain positions, although most of the sequence is conserved (19). Synthetic peptides corresponding to one variable region elicited the formation of low levels of neutralizing antibody.

Predictions from Reaction of Virus with Neutralizing Monoclonal Antibody

The value of monoclonal antibodies in locating immunogenic sites has been demonstrated elegantly by Minor and his colleagues (41) with poliovirus, type 3. As with foot-and-mouth disease virus, the major immunizing sites appear to be on VP1. By passaging the virus in the presence of neutralizing monoclonal antibodies, naturally occurring mutants in the virus population were selected for their resistance to neutralization. Comparison of the original and mutant viruses by ribonuclease T1 mapping of their RNAs showed that a specific oligonucleotide was missing from the mutant strains. This oligonucleotide was about 300 nucleotides from the 5' end of the gene coding for VP1.

Further nucleotide sequencing in this region of the RNA shows that most of the mutations conferring resistance to neutralization are confined to a tract of eight amino acids coded for by residues 277–300. A closely analogous region of VP1 of poliovirus type 1 had also been shown to be a neutralization epitope (68). A peptide covering this region of type 3 virus, when coupled to bovine thynglobulin, elicits neutralizing antibodies in rabbits (21). This region is the same as that shown by Emini et al (19) to evoke a neutralizing antibody response.

Predictions from Secondary Structure

It would be clearly of great value to be able to predict whether a given amino acid sequence is on the surface of a protein and therefore able to react with the immune system. The most precise predictions would require a knowledge of the structure of a virus at the atomic level. This knowledge is not available for more than a few viruses, so recourse has been made to other methods. Because it is generally accepted that tracts containing the hydrophilic amino acids

lysine, arginine, aspartic acid, and glutamic acid are more likely to be on the surface of a protein, locating these in the amino acid sequence will provide a useful guide in the prediction of likely immunogenic sites. This method is necessarily imprecise because there can be no certainty that some of these sites are not involved in the interaction of the immunizing protein with the nucleic acid and other proteins of the virus.

Nevertheless hydrophilic plots have proved useful in predicting immunogenic sites. Thus the 134–160 region of VP1 of foot-and-mouth disease virus is both hydrophilic and immunogenic, but the same protein also possesses six other hydrophilic regions, none of which appears to possess the immunogenicity of the 134–160 region. It was the combination of predictions from the biological activity of fragments in that region and the high variability of the amino acid squence that allowed the correct prediction to be made.

Several predictions have also been made of the active region of the surface protein of hepatitis B virus. Lerner's group (34) synthesized peptides corresponding to different regions of the protein and tested the antibodies they elicited for their ability to react with the native protein. Peptides that were very hydrophobic and those of six or fewer residues were ineffective, but those that were longer and soluble were effective, especially if they contained proline. This amino acid is particularly important because it occurs at "corners," which means that the amino acids on either side are near each other, thus producing a minor conformational determinant. In addition, proline residues have an imide rather than an amide bond, so that several atoms surrounding this residue have a three-dimensional relationship to one another, whether in a protein or a peptide, and will therefore be recognized by antibody in either situation.

The same group of workers (24) subsequently showed that a peptide containing residues 95–109 induced virus-neutralizing antibody in chimpanzees and afforded protection against challenge. Other groups using prediction methods have also found that peptides from nearby regions of the protein are active (7, 17, 28, 50).

Predictions have also been made with other viruses. Influenza virus has attracted most attention but the situation is far from clear. Lerner's group (60) showed that peptides corresponding to regions of the virus hemagglutinin not included in the four antigenic domains would elicit antibodies that reacted with the intact protein. This observation gave rise to the attractive concept that a peptide corresponding to a conserved sequence common to different strains of the virus might be used as a "universal" vaccine. Arnon and her colleagues (46) have found that a synthetic peptide corresponding to amino acids 91–108 of the hemagglutinin will protect mice against challenge. This tract, which is common to at least nine strains of the virus, comprises a folded region in the polypeptide chain and contains three tyrosine and two proline residues. From this information it was predicted that it would be immunologically active, and the biological results are encouraging.

Clearly predictions based on secondary structure are highly speculative, and no rules have so far emerged that are particularly useful on their own in determining the location of immunogenic sites. Equally clearly, as the activity of more peptides is tested, useful rules will emerge.

PRIMING WITH PEPTIDES

The most exciting observation to be made recently with virus antigens is that by Emini et al (19) with poliovirus peptides. They showed that rabbits that had received peptide corresponding to the antigenic site in amounts insufficient to elicit a measurable level of neutralizing antibody produced very high levels of neutralizing antibody when injected with small amounts of poliovirus particles. Similar amounts of polio particles did not elicit measurable levels of neutralizing antibody in unprimed animals.

The priming effect has also been found with other viruses, and there seems to be no reason why it should not apply universally. Extension of the observation should throw considerable light on the structural requirements for immunogenicity. In addition, the potential of the approach in potentiating the activity of antigens would seem to be very great indeed.

PROSPECTS FOR THE FUTURE

The capability of cloning and sequencing nucleic acids now means that it is possible to synthesize, either chemically or biologically, almost any virus peptide or protein. In the case of peptides it is possible not only to synthesize directly the relevant parts of naturally occurring sequences but also to produce "artificial peptides" that may have properties superior to those of the naturally occurring sequences. Similarly, artificial proteins can be produced by point site mutation of the corresponding DNA. The greatest advantage at present, however, would seem to stem from the capability of synthesizing proteins that cannot be produced conventionally because of our inability to grow the viruses to the levels necessary for the production of vaccines.

A major problem that still confronts us is the difficulty of ensuring that the conformation of the engineered proteins mimics that of the antigenic proteins in their natural environment. This difficulty stems from our lack of knowledge of the conformation of the antigenic proteins on the virus particles. At present this can only be determined by X-ray crystallography, which is both time-consuming and costly. However, there are no short cuts, and any advances that are made with individual proteins will be fortuitous rather than based on a fundamental understanding of the factors determining configuration. With the increasing interest in this aspect of the problem that has stemmed from a knowledge of the sequences of several immunogenic proteins, rapid advances are likely in the next few years.

Literature Cited

1. Almeida, J. D., Waterson, A. P. 1970. *Arch. Ges. Virusforsch.* 32:66–72
2. Anderer, F. A. 1963. *Z. Naturforsch. Teil B* 18:1010–14
3. Anderer, F. A. 1963. *Biochim. Biophys. Acta* 71:246–48
4. Andrewes, C. H., Horstmann, D. M. 1949. *J. Gen. Microbiol.* 3:290–97
5. Bachrach, H. L., Moore, D. M., McKercher, P. D., Polatnick, J. 1975. *J. Immunol.* 115:1636–41
6. Barin, F., Maupas, P., Coursaget, P., Goudeau, A., Chiron, J. P. 1981. In *INSERM Symp. 18*, ed. P. Maupas, P. Guestry. 263–66. Paris: Inst. Natl. Santé Rech. Méd.
7. Bhatnagar, P. K., Papas, E., Blum, H. E., Milich, D. R., Nitecki, D., et al. 1982. *Proc. Natl. Acad. Sci. USA* 79: 4400–04.
8. Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., et al. 1982. *Nature* 298:30–33
9. Boothroyd, J. C., Highfield, P. E., Cross, G. A. M., Rowlands, D. J., Lowe, P. A., et al. 1981. *Nature* 290:800–2
10. Brammar, W. J. 1982. In *Genetic Engineering*, ed. R. Williamson, 3:53–81
11. Brenner, S., Horne, R. W. 1959. *Biochim. Biophys. Acta* 34:103–10
12. Brown, F., Cartwright, B., Smale, C. J. 1967. *J. Immunol.* 99:171–77
13. Deleted in proof
14. Crick, J., Brown, F. 1969. *Nature* 222:92
15. Davenport, F. M., Hennessy, A. V., Brandon, F. M., Webster, R. G., Barrett, C. D., et al. 1964. *J. Lab. Clin. Med.* 63:5–13
16. Davis, A. R., Bos, T., Veda, M., Nayak, D., Dowbenko, D. Compans, R. W. 1983. *Gene* 21:273–84
17. Dreesman, G. R., Sanchez, Y., Ionescu-Matiu, I., Sparrow, J. T., Six, H. R., et al. 1982. *Nature* 295:158–60
18. Dubois, M. F., Pourcel, C., Rousset, S., Chany C., Tiollais, P. 1980. *Proc. Natl. Acad. Sci. USA* 77:4549–53
19. Emini, E. A., Jameson, B. A., Wimmer, E. 1983. *Nature* 304:699–703
20. Emtage, J. S., Tacon, W. C. A., Catlin, G. H., Jenkins, B., Porter, A. G., et al. 1980. *Nature* 283:171–74
21. Ferguson, M., Minor, P. D., Magrath, D. I., Qi Y.-H., Spitz, M., Schild, G. C. 1984. *J. Gen. Virol.* 65:197–201
22. Fujisawa, Y., Ito, Y., Sasada, R., Ono, Y., Igarashi, K., et al. 1983. *Nucl. Acids Res.* 11:3581–91
23. Gard, S., Carlstrom, G., Lagercrantz, R., Norrby, E. 1964. *Arch. Virusforsch.* 16:315–23
24. Gerin, J., Alexander, H., Shih, J.-W. K., Purcell, R. H. Dapolito, G., et al. 1983. *Proc. Natl. Acad. Sci.* 80:2365–69
25. Gething, M. J., Sambrook, J. 1981. *Nature* 293:620–25
26. Gething, M. J., Sambrook, J. 1982. *Nature* 300:598–603
27. Hitzeman, R. A., Chen, C. Y., Hagie, F. E., Patzer, E. J., Liu, C. C., et al. 1983. *Nucl. Acid. Res.* 11:2745–63
28. Hopp, T. P., Woods, K. R. 1981. *Proc. Natl. Acad. Sci. USA* 78:3824–28
29. Kaaden, O. R., Adam, K.-H., Strohmaier, K. 1977. *J. Gen. Virol.* 197:397–400
30. Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., et al. 1981. *Science* 214:1125–29
31. Kupper, H., Keller, W., Kurz, C., Forss, S., Schaller, H., et al. 1981. *Nature* 289:555–59
32. Langbeheim, H., Arnon, R., Sela, M. 1976. *Proc. Natl. Acad. Sci. USA* 73: 4636–40
33. Laporte, J., Grosclaude, J., Wantyghem, J., Bernard, S., Rouze, P. 1973. *C. R. Acad. Sci. Paris* 276:3399–401
34. Lerner, R. A., Green, N., Alexander, H., Liu, F. T., Sutcliffe, J. G., et al. 1981. *Proc. Natl. Acad. Sci. USA* 78: 3403–07
35. Maassab, H. F. 1967. *Nature* 213:612–14
36. Mackett, M., Smith, G. L., Moss, B. 1982. *Proc. Natl. Acad. Sci. USA* 79: 7415–19
37. Marquardt, O. 1982. *J. Gen. Virol.* 59: 283–94
38. Matthews, R. E. F. 1982. *Intervirology* 17:33–199
39. McAleer, W. J., Buynak, E. B., Maigetter, R. Z., Wampler, D. E., Miller, W. J., Hilleman, M. R. 1984. *Nature* 307: 178–80
40. Meloen, R. H., Rowlands, D. J., Brown, F. 1979. *J. Gen. Virol.* 45:761–63
41. Minor, P. D., Schild, G. C., Ferguson, M., MacKay, A., Magrath, D. I., et al. 1982. *J. Gen. Virol.* 61:167–76
42. Moore, D. M. 1983. In *Genetic Engineering: Applications to Agriculture, Beltsville Symposium*, ed. L. D. Owens, 7:89–106. Totowa, NJ: Rowman & Allanheld
43. Morein, B., Helenius, A., Simons, K., Pettersson, R., Kaariainen, L., et al. 1978. *Nature* 276:715–18

44. Morein, B., Sharp, M., Sundquist, B., Simons, K. 1983. *J. Gen. Virol.* 64:1557–69
45. Moriarty, A. M., Hoyer, B. H., Shih, J. W.-K., Gerin, J. L., Hamer, D. H. 1981. *Proc. Natl. Acad. Sci. USA* 78:2606–10
46. Muller, G. M., Shapira, M., Arnon, R. 1982. *Proc. Natl. Acad. Sci. USA* 79: 569–73
47. Myanohara, A., Tobe, A., Nozaki, C., Hamada, F., Ohtomo, N., et al. 1983. *Proc. Natl. Acad. Sci. USA* 80:1–5
48. Panicali, D., Paoletti, E. 1982. *Proc. Natl. Acad. Sci. USA* 79:4927–31
49. Pfaff, E., Mussgay, M., Bohm, H. O., Schulze, G. E., Schaller, H. 1982. *EMBO. J.* 1:869–74
50. Prince, A. M., Ikram, H., Hopp, T. P. 1982. *Proc. Natl. Acad. Sci. USA* 79: 579–82
51. Robson, K. J. H., Doel, T. R., Gorman B. M., Brown, F. 1980. *J. Gen. Virol.* 46:179–93
52. Rose, J. K. Shafferman, A. 1978. *Proc. Natl. Acad. Sci. USA* 78:6670–74
53. Rowlands, D. J., Bittle, J. L., Houghten, R. A., Lerner, R. A., Brown, F. Unpublished observations
54. Sabin, A. B. 1956. *J. Am. Med. Assoc.* 163:1582–96
55. Sangar, D. V. 1979. *J. Gen. Virol.* 45:1–13
56. Skelly, J., Howard, C. R., Zuckerman, A. 1981. *Nature* 390:51–54
57. Smith, G., Mackett, M. A., Moss, B. 1983. *Nature* 302:490–95
58. Stanway, G., Cann, A. J., Hauptmann, R., Mountford, R. C., Clarke, L. D., et al. 1983. *Eur. J. Biochem.* 135:529–33
59. Strohmaier, K., Franze, R., Adam, K.-H. 1982. *J. Gen. Virol.* 59:295–306
60. Sutcliffe, J. G., Shinnick, T. M., Green, N., Lerner, R. A. 1983. *Science* 39:660–66
61. Sveda, M. M., Lai, C. J. 1981. *Proc. Natl. Acad. Sci. USA* 78:5488–93
62. Sveda, M. M., Markoff, L. J., Lai, C. J. 1982. *Cell* 20:649–56
63. Theiler, M., Smith, H. 1937. *J. Exp. Med.* 65:767–86
64. Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., Hall, B. D. 1983. *Nature* 298:247–350
65. Watson, R. J., Weis, J. H., Salstrom, J. S., Enquist, L. W. 1983. *Science* 218: 281–384
66. Wild, T. F., Burroughs, J. N., Brown, F. 1969. *J. Gen. Virol.* 4:313–20
67. Winther, M. D., Allen, G., Bomford, R. H., Brown, F. Unpublished observations
68. Wychowski, C., van der Wer, S., Siffert, O., Crainic, R., Bruneau, P., et al. 1983. *EMBO Journal* 2:2019–24
69. Yelverton, E., Norton, S., Obijeski, J. F., Goeddel, D. V. 1983. *Science* 219: 614–19

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ALTERATIONS IN OUTER MEMBRANE PERMEABILITY

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INTRODUCTION

The outer membrane serves as a physical and functional barrier for gram-negative bacteria. In recent years, considerable efforts have been made to understand the functions, synthesis, chemical composition, and structure of outer membrane components (34, 84, 101, 102, 119, 129). Parallel with these studies, a number of experiments have been addressed to integrating these data in an attempt to understand the functioning of the outer membrane as a whole. This review will examine those studies that concentrate on alterations of the outer membrane barrier properties and will attempt to explain these permeability alterations on the basis of a single class of critical outer membrane sites. To provide a framework for discussion, the structure and permeability properties of wild-type outer membranes will first be briefly discussed.

Structure of the Cell Envelope

The gram-negative cell envelope consists of two membranes separated by a layer of peptidoglycan and a cellular compartment called the periplasm. The innermost, cytoplasmic membrane is generally a phospholipid bilayer liberally studded with a wide variety of polypeptides. The major functions of cytoplasmic membrane proteins are in energy generation, in active and facilitated transport of nutrients and export of toxic byproducts, and in enzymatic synthesis and translocation of cell envelope components (148). The cytoplasmic membrane serves as a major barrier for hydrophilic or charged molecules (in the absence of a utilizable transport system), but it is generally accepted that even moderately hydrophobic molecules can enter into or even cross the lipid bilayer at growth temperatures (117).

The periplasm (previously called the periplasmic space) is probably a matrix of polypeptides and saccharides with net negative charges (89, 171). It contains a variety of enzymes, some of which function as scavenger or processing enzymes for conversion of nontransportable metabolites to transport substrates. Little is known about the barrier function of the periplasm, nor, for that matter, of the peptidoglycan that serves as a primary shape and osmotic stability–maintaining determinant of the cell.

The outer membrane is an unusual biological membrane in that its outer monolayer contains lipopolysaccharide (LPS) as its major lipidic molecule, while the inner leaflet contains phospholipids rather than LPS (102, 119). LPS is an amphiphilic molecule containing a hydrophobic region (Lipid A, also known as endotoxin) that has 5 or 6 fatty acids linked to diglucosamine phosphate. Covalently attached to this is the rough oligosaccharide core containing in its proximal portion an unusual sugar, 2-keto-3-deoxyoctanate (KDO), as well as a variety of heptose and hexose residues (48, 102). The rough oligosaccharide core may be substituted with a variable number of repeated tri- to penta-saccharide units called the O-antigen (132).

The LPS carries a net negative charge resulting in the strong negative surface charge of gram-negative cells (16, 162). One of the most important features of LPS is that it appears to be anchored in the outer membrane by binding to outer membrane proteins (159, 212), possibly through hydrophobic interactions with Lipid A (212), and by noncovalent cross-bridging of adjacent LPS molecules with divalent cations (61, 98, 102, 119). Thus treatment of gram-negative cells with ethylenediaminetetraacetate (EDTA) generally results in removal, by chelation, of divalent cations and consequent disruption of the outer membrane (see below).

In the absence of such chelators, however, the combination of negative charge and divalent cation cross-bridging of LPS provides gram-negative cells with many of their more important properties, including resistance to hydrophobic antibiotics, bile salts, detergents, proteases, lipases, and lysozyme (74, 98, 102, 117, 119). The outer membrane also contains a small number of so-called "major" proteins present in high copy number (10^5 copies per cell) (102). These proteins may have structural roles in that they anchor, either covalently [in the case of Braun's lipoprotein (14)] or more usually noncovalently (102), the outer membrane to the underlying peptidoglycan. Little is known about the function of outer membrane proteins with the exception of porins that form relatively nonselective, water-filled channels of defined exclusion limits for hydrophilic compounds (9, 119).

Recent studies have defined structural discontinuities in outer membranes that appear in freeze fracture electron microscopic studies as particles and pits on opposing fracture faces of the outer membrane (102). These particles are 4–8 nm in diameter and probably contain both proteins and lipopolysaccharide. As many as 60,000 particles may be present in the outer membrane of a single cell. Of interest to this review is that some of the particles probably contain the chelator-sensitive sites of the outer membrane, in that EDTA and Ca^{2+} can markedly influence the observed number of such sites (102, 197). In addition, evidence has been presented that suggests that some of these sites contain porin proteins (102).

Uptake Across the Outer Membrane

THE HYDROPHILIC PATHWAY It is now well established that hydrophilic compounds can pass across the outer membrane via the water-filled channels of proteins, called "porins" (9, 119). The channel area of individual porin pores and therefore the effective exclusion limit of porins and of outer membranes varies from organism to organism. For example, *Escherichia coli* allows the passage of trisaccharides (119) or tetrapeptides (133), whereas *Pseudomonas aeruginosa* (72) and *Neisseria gonorrhoeae* porins (37) exclude only those saccharides of molecular weights greater than about 6000. The rate of uptake, by different porins, of different hydrophilic compounds is determined by the molecular properties of the porin channel, with channel size and ionic selectiv-

ity relative to the size and charge of the compound (9, 119, 120) as well as the total number of available channels per cell (2, 114) playing major roles.

In addition, because porin channels are filled with water and have charged amino acid residues at the mouth of or within the channel (9), solute passage is a function of the intrinsic viscosity (i.e. the hydrophobicity) of the permeating molecule (120). The movement of small, very hydrophobic compounds through porin channels will be severely restricted, as demonstrated experimentally by Nikaido (117).

THE HYDROPHOBIC PATHWAY Many lipid bilayer membranes allow the passive uptake of hydrophobic compounds into the membrane interior and the passage of amphiphilic (moderately hydrophobic) molecules across the membrane (e.g. 160, 169). In contrast, Nikaido (117) has demonstrated that the outer membranes of wild-type *Salmonella typhimurium* and *E. coli* cells do not allow the passage of hydrophobic or amphiphilic molecules. Symptomatic of the lack of a hydrophobic uptake pathway is cellular resistance to hydrophobic antibacterial agents like actinomycin D, phenol, and crystal violet, as well as resistance to detergents and bile salts (119).

Because this is a common property of many wild-type gram-negative bacteria, with the possible exception of some *Neisseria* strains (see below), it would seem that the hydrophobic uptake pathway is very inefficient in gram-negative bacteria in contrast to some but not all gram-positive bacteria (46). Even in mutants of *Salmonella* with heptose-deficient LPS, which were shown by Nikaido to have a hydrophobic uptake pathway (117), the pathway can apparently be blocked by the addition of divalent cations (85, 168). This and other data suggest that the combined effects of divalent cation bridging of LPS molecules and high-surface negative charge may be responsible for the absence of a hydrophobic uptake pathway in most gram-negative bacteria.

THE SELF-PROMOTED PATHWAY The self-promoted pathway has been postulated for the uptake of polycationic antibiotics, like polymyxins and aminoglycosides, across the outer membrane of *P. aeruginosa* (70, 74, 75). It involves the displacement of divalent cations from LPS by these polycations, thus destroying the LPS cross-bridging and destabilizing the outer membrane (74, 75). Because this can result in enhancement of uptake of lysozyme, β-lactams (75), and hydrophobic fluorescent dyes (100) across the outer membrane, we have proposed that such interactions promote the uptake of the interacting polycationic antibiotic itself.

As further evidence in favor of self-promoted uptake, EDTA, a divalent cation chelator that removes Mg^{2+} from outer membrane sites, causes similar enhancement of uptake of lysozyme and β-lactams (74) as well as enhanced killing by the polycationic antibiotics (177). Furthermore, a single point mutation in *P. aeruginosa* renders the cell resistant to not only the polycationic antibiotics, but also EDTA (113), while external Mg^{2+} inhibits the action of both classes of agents (112, 216).

Although the self-promoted pathway has not been demonstrated in other bacteria, the similarity of polymyxin and EDTA effects on many bacteria, including *P. aeruginosa* (see below), suggests it may well be a common pathway of uptake. Interestingly, a number of the membrane-active agents effective against gram-negative bacteria are cationic, including the quaternary ammonium disinfectants, chlorhexidine and other diguanidine compounds, gramicidin S and tyrocidin, as well as the polymyxins.

Intrinsic Defects in Outer Membrane Permeability in Some Bacteria

The opportunitistic pathogen *Pseudomonas aeruginosa* demonstrates intrinsic resistance to a wide range of antibiotics (17). It has now been clearly demonstrated that this is due to the poor permeability of its outer membrane (2, 114, 214). Despite the fact that the major porin of *P. aeruginosa* forms substantially larger channels than enteric organisms (8, 72), its activity is substantially lower, and it has been estimated that only 0.2–1% of the available porin molecules form open functional channels (2, 114).

Because the rate of diffusion of hydrophilic compounds is proportional to the total available area of water-filled porin channels across the outer membrane, the rate of uptake of all hydropilic compounds is reduced (114). Presumably this property of *P. aeruginosa* is of advantage in its major ecological niche, the soil, in which many antibiotic-secreting organisms compete. Although similar molecular studies have not been performed on other soil-derived, pathogenic pseudomonads, their general property of high intrinsic antibiotic resistance (42) is suggestive of a common defect in outer membrane permeability.

In contrast to other gram-negative bacteria, some organisms, notably *N. gonorrhoeae*, demonstrate higher uptake of crystal violet (210) and high susceptibility to hydrophobic agents such as erythromycin, rifampicin, acridine orange, ethidium bromide, and free fatty acids (151). This has lead to the proposal that these bacteria have a functioning hydrophobic uptake pathway (119).

MUTATIONAL ALTERATIONS IN OUTER MEMBRANE PERMEABILITY

A large number of potential mutants altered in outer membrane permeability have been isolated. One of the primary problems in assigning the mutational defects in these mutants is the lack of specific experiments demonstrating that the mutants have outer membrane permeability alterations. Relatively unam-

biguous techniques for assessing outer membrane permeability changes include hydrolysis of β-lactams by periplasmic β-lactamase in whole cells [using the methodology of Zimmerman & Rosselet (217)] as a measure of hydrophilic permeability and uptake of gentian violet (210) as a measure of hydrophobic permeability.

Often, however, more ambiguous means are employed. Generally speaking, I have considered as mutants in outer-membrane permeability those strains with clearly defined biochemical alterations in the outer membrane together with enhanced susceptibility to antibiotics, dyes, chelators, or detergents. In addition, given the importance of the outer membrane as a barrier to moderately hydrophobic antibiotics (see above) and detergents, any mutants altered in susceptibility to these compounds must be considered potential outer membrane permeability mutants. A more detailed summary of the properties of the individual outer membrane permeabilty mutants is contained in Table 1.

Porin-Deficient Mutants

Porin-deficient mutants have been isolated in a variety of *Enterobacteriaceae* and in *P. aeruginosa*. *E. Coli* porin-deficient mutants are specifically altered in the uptake across the outer membrane of hydrophilic molecules, including a variety of β-lactam antibiotics (78, 87, 91, 152), tetracyclines (45), chloramphenicol (45), nucleotides (4, 195), methionine (103), saccharides (3), Ca^{2+}, and silver (103). For the smaller substrates mentioned above, these effects manifest themselves at low substrate concentrations when outer-membrane permeation is rate-limiting. Porin-deficient mutants grow relatively normally at higher external concentrations, where the diffusion rate across the outer membrane creates a periplasmic concentration of substrate substantially higher than the affinity constant (Km, Kd) for transport or binding of the substrate.

Measurement of the rate of permeation of β-lactams into porin-deficient mutants has demonstrated a 10- to 100-fold lower rate of permeation into these strains (3, 114, 118, 121, 122, 130). This suggests that the total area of porin channels in the outer membrane is reduced to 1–10%: presumably the residual uptake of hydrophilic substrates is due to alternative (7) or residual (3) porin molecules. A single bacterium can have up to five different porins. For example, *E. coli* has the OmpC, OmpF (121), LamB (11), PhoE (121), and protein K (205) porins. The molecular properties of these porins and their activities (i.e. proportion of open functional channels) can vary considerably (9, 11).

Thus, deletion of one of these porins by mutation is not necessarily equivalent to deletion of another. For example, *ompF* mutants have greater alterations in the uptake of nucleotides than *ompC* mutants (195), leading to the erroneous conclusion that OmpF pores are "specific" for nucleotides. It is now known that the real reason for this observation is the lower activity and/or smaller size of the OmpC protein pore (121). However, mutations in alternative porins can result in a specific alteration in the permeability of the outer membrane; for example, maltose and maltodextran transport are preferentially reduced in mutants deficient in the Lam B porin protein (11).

While substantial alterations in the outer membrane permeability and, consequently, the resistance of cells to hydrophilic antibiotics are observed in porin-deficient mutants, the sensitivity of cells to polycationic (91, 110) and hydrophobic antibiotics and detergents (83) is unaltered.

Pseudomonas aeruginosa wild-type cells have a 12-fold lowered permeability to β-lactam antibiotics compared to *E. coli* (114, 214) and a consequent higher resistance to hydrophilic antibiotics (17). Therefore, wild-type *P. aeruginosa* cells behave like porin-deficient mutants despite the high copy number of their major porin protein F (2, 114). This is primarily due to the low activity of this porin [only 0.4% of the protein F in the outer membrane forms pores (114)]. Loss of protein F by mutation decreases the outer membrane permeability to β-lactams by six-fold (114) but has only a small effect on sensitivity to many antibiotics (112a).

Adaptation and Resistance to Antibiotics

An effective method of isolating porin-deficient mutants in the *Enterobacteriaceae* is by selection for resistance to mono- or di-anionic β-lactams (78, 87, 91, 152). Alternatively, such mutants can be selected as resistant to low levels of chloramphenicol (45). The *pen*B2 mutant of *N. gonorrhoeae* results in low-level resistance to hydrophilic antibiotics, possibly due to a porin alteration (67).

Outer membrane–altered mutants, resistant to polymyxin B, have been isolated in both *P. aeruginosa* (113) and *S. typhimurium* (105, 191). In both cases, cells become refractory to the effects of EDTA (113, 187, 192). In addition, the mutants are cross resistant to polycationic compounds like aminoglycosides (for *P. aeruginosa*), protamine, and polylysine (for *E. coli*). The permeability of the outer membrane towards β-lactam antibiotics is, however, unaltered (114, 185). In each case, the characterized mutational defect is consistent with these cells having a defective self-promoted uptake pathway. The *P. aeruginosa* polymyxin B resistant mutants have an increased level of a major outer membrane protein H1 and a corresponding decrease in cell envelope Mg^{2+} (113). This led us to propose that protein H1 may replace Mg^{2+} (presumably via protonated amino groups) at LPS Mg^{2+}-crossbridging sites that were proposed to be the sites of interaction of polymyxin B, aminoglycosides, and EDTA with the cell surface (70, 113, 114).

In support of this, a phenotype identical to the one appearing in these polymyxin resistant mutants is acquired by wild-type cells growing in Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}-deficient medium and reversed by addition of any of these

Table 1 Mutants affecting outer membrane permeability[a]

Mutant	Primary alteration	Outer membrane hydrophilic permeability	Gentian violet uptake	Alterations in susceptibility[b]					References
				DET	EDTA	HPI	HPO	PCAT	
Porin^{-} [c]	porin-deficient	6–100X ↓	—	U	—	R	U	U	3, 4, 45, 75, 78, 83, 87, 91, 103, 110, 114, 118, 121, 130, 152, 195
PX^{r} [d]	OM protein H1 over-produced	U	—	U	R	U	U	R	113–115
*pmr*A[e]	LPS Lipid A	U	—	SS	R	U	U	R	105, 185, 187, 191, 192
mtr	—	—	↓	R	—	R	R	U	66, 104, 166
Z61[d]	LPS Lipid A	6X ↑	—	—	—	SS	SS	SS	2, 93
1–4[d]	—	↑	—	SS	—	SS	U	SS	128, 141
*acr*A[g]	Lipid A phosphate	—	—	SS	—	SS	SS	SS/U	26, 111
*env*A[g]	LPS (?)	4.5X ↑	↑	U	—	SS	SS	SS	64, 126, 127
*env*B,M–T[g]	—	—	—	SS	—	SS	SS	SS	38, 125
env[f]	—	—	↑	SS	—	SS	SS	—	66, 151
DC2[g]	—	↑	—	—	—	SS	SS	—	140
H101[g]	—	—	—	SS	—	U	SS	—	40
lky[c,g]	periplasmic-leaky mutants	—	—	SS/U	SS/—	SS	SS	SS/—	1, 96
*tol*A,B[g]	colicin tolerant	—	—	SS	SS	SS	SS	—	10, 30
*tol*C[g]	TolC OM protein	—	—	SS	SS	SS	SS	—	30, 109
*tol*D,E[g]	LPS	—	↑	SS	—	—	—	—	41
*rfa*E[c,g]	heptose-deficient LPS	—	↑	SS	SS	U	SS	SS/U	6, 76, 85, 88, 117, 142, 149, 156–158, 163, 168, 178, 193, 194, 210
*rfa*D[e]	LPS	—	—	SS	—	U	SS	—	12
*lps*A[g]	LPS	—	↑	SS	—	—	—	—	65
*rfa*H[c,g]	leaky LPS rough mutation	—	—	—	—	—	SS	SS	150
*rfa*Rd[e]	glucose-deficient LPS	—	—	SS	—	U	SS	SS	76, 134
AK43[d]	rough LPS	—	—	—	—	SS	SS	SS	92
pss[g]	PSS-deficient	—	—	—	—	SS	SS	SS	135a
lpo[g]	Braun lipoprotein (BLP)	U	—	SS	SS	U	SS	—	7, 176, 213
*lky*D[e]	PG-bound BLP	—	—	SS	SS	—	SS	—	47, 201

[a]Abbreviations used: LPS = lipopolysaccharide; OM = outer membrane; PSS = phosphatidyl serine synthetase; BLP = Braun's lipoprotein; PG = peptidoglycan.

[b]Abbreviations for classes of compounds: DET = detergents; EDTA = ethylenediaminetetraacetate; HPI = hydrophilic antibiotics; HPO = hydrophobic antibiotics; PCAT = polycationic compounds. R signifies resistant; SS = supersensitive; U = unaltered susceptibility.

[c,d,e,f,g]Denotes the bacterial species in which the mutant was isolated, as follows: c = many different species; d = *Pseudomonas aeruginosa;* e = *Salmonella typhimurium;* f = *Neisseria gonorrhoeae;* g = *Escherischia coli.*

four cations to the growth medium (115). In the case of the *S. typhimurium* *pmr*A mutant, an increase in the amount of 4-amino-4-deoxy-L-arabinose bound to Lipid A phosphate in the LPS (191) would convert the negative charge on phosphate to a positive charge on the arabinosamine. This might reduce Mg^{2+}-crossbridging of the LPS due to the formation of ion pairs between the arabinosamine and an adjacent phosphate (rather than Mg^{2+}-crossbridging between phosphates on adjacent LPS molecules). Such an ion pair should not be attacked by polymyxin or EDTA.

The *E. coli* polymyxin-resistant mutants *(pmx)* of Dame & Shapiro are not well characterized biochemically. However, they are quite sensitive to detergents and hydrophobic antibiotics. Since *pmr*A and *pmx* mutants share susceptibility to sodium deoxycholate and resistance to polymyxin B (29, 105), it may well be that these mutants are analagous or identical.

Seratia marcescens and *P. aeruginosa* strains grown in increasing concentrations of polymyxin B become adapted to high levels of polymyxin, in that resistance is dependent on the presence of polymyxin B and reverts rapidly upon growth of the strains in the absence of polymyxin B (52). These strains apparently develop multiple outer membrane changes (51), and many different explanations have been forwarded for this adaptation to polymyxin B. One possibility is the cells are able to establish a stable state in which polymyxin B is bound to or incorporated into the outer membrane (182). In any case, *S. marcescens* and *P. aeruginosa* adapted to polymyxin B become cross-resistant to polycationic compounds, but hypersensitive to detergents and rifampicin (52, 182).

The reason for this latter observation is probably the action of polymyxin B (which must be present during growth to stabilize the adaptation) on the outer membranes to alter the detergent susceptibility, as shown for a naturally polymyxin-resistant isolate of *Proteus mirabilis* (173). In addition, polymyxin B apparently stimulates the action of a phospholipase (23) [perhaps phospholipase A1 (94) of the outer membrane] in polymyxin-adapted strains. How these cells manage to maintain a stable state in which they interact with polymyxin B but are able to grow in its presence is an unresolved paradox worthy of further investigation.

Antibiotic-Supersensitive Mutants

A number of mutants have been described to be supersensitive to a wide variety of antibiotics (Table 1). Mutant Z61 of *P. aeruginosa* is 4- to 10,000-fold more susceptible to 30 different antimicrobial agents (2). This strain has a 6-fold enhancement in outer membrane permeability to the β-lactam, nitrocefin (2), although there is no apparent alteration in the major porin protein. Instead, mutant Z61 has an alteration in lipid A, and we have proposed that the altered LPS of mutant Z61 interacts with porin and favors a higher percentage of open,

functional pores (93). Similar, but less well-characterized mutants of *P. aeruginosa* have been described (128, 141).

E. coli strains selected as supersensitive to acridine orange, *acr*A, are also more sensitive than wild-type cells to six hydrophobic antibiotics and two detergents (111). The *acr*A mutation apparently results in a reduction in lipid A phosphate (26). This mutation could be explained if the reduction in critical anionic sites involved in stabilization of the outer membrane by divalent cations resulted in a less stable outer membrane that could allow increased uptake of hydrophobic compounds.

Other biochemically undefined mutants of *E. coli* resulting in enhanced sensitivity to hydrophobic antibiotics have been described. These have been isolated as having enhanced susceptibility to various agents (38, 40, 125) or as periplasmic-leaky mutants that secrete various periplasmic components (see below). One of the best characterized of the former class, the *env*A mutant, has enhanced permeability to β-lactams and to gentian violet and enhanced susceptibility to a wide variety of antibiotics and detergents. It is thought to have a subtle LPS alteration (64), although it is difficult to reconcile this with its tendency to form long filiaments (126). The *env* mutation of *N. gonorrhoeae* results in hypersensitivity to a variety of hydrophobic and hydrophilic antibiotics and enhanced uptake of gentian violet (66, 151). In contrast, the *mtr* locus (also called *ery*) encodes the inverse phenotype (i.e. resistance to the same hydrophobic and hydrophilic antibiotics and reduced uptake of gentian violet) (66, 104, 166).

LPS Alterations

Perhaps the best-studied example of mutational alterations in outer membrane permeability is afforded by deep-rough (heptose-deficient) mutants of *E. coli* and *S. typhimurium* (Table 1). These mutants demonstrate enhanced sensitivity to a variety of hydrophobic antibiotics and detergents (Table 1). In addition, they demonstrate enhanced uptake of the hydrophobic dye gentian violet (112, 168), enhanced susceptibility to EDTA (163), Tris-lysozyme (8, 163), and phospholipases (57, 88), and leakage of periplasmic enzymes (163, 193). Despite this leakiness to water-soluble periplasmic enzymes, deep-rough mutants do not have enhanced susceptibility to hydrophilic compounds (78, 157, 158) (although no direct measurements of hydrophilic permeability have been performed).

The biochemical nature of the surface of deep-rough organisms is the source of considerable controversy. The molar ratios of phospholipids to LPS that are present in the outer membrane have been described as varying between 2.5:1 and 8.9:1 (mean = 5.0:1) for wild-type strains and between 1.2:1 and 4.4:1 (mean = 3.3:1) for deep-rough (*rfa*E) mutants (57, 58, 102, 161, 164). It

should be noted that each of the above studies employs many assumptions, the major of which is that extracted outer membranes have a composition identical to native outer membranes, an assumption of doubtful validity. From their data, Smit & Nikaido have suggested that deep-rough mutants have patches of exposed phospholipids on their surfaces, resulting in areas of phospholipid bilayer in the outer membrane (164).

In contrast, the data of Gmeiner & Schlecht (57, 58) suggests that the asymetric nature of the outer membrane (LPS in the outer monolayer, phospholipids in the inner) may be maintained in deep-rough mutants. The susceptibility of phospholipids in deep-rough strains to phospholipase digestion (57, 88) has been cited in favor of the former model, but given the permeability of deep-rough mutants to proteins, including lysozyme and periplasmic proteins (85, 163, 193), it seems possible that these outer membranes may also be permeable to phospholipases. One piece of evidence in favor of the latter model is the reversal of the phenotype of deep-rough mutants by Mg^{2+}. It might be that the higher amount of LPS in deep-rough strains [as reported by two groups (57, 58, 161) but not by Smit & Nikaido (164)] brings about a higher requirement of these cells for Mg^{2+} in order to stabilize their outer membranes.

Furthermore, the effects of polymyxin, which extracts Mg^{2+} (but little LPS) (115, 189) from the outer membrane mimic the effects of the deep-rough mutation (see Tables 1 and 2). Thus, while I personally favor the concept of maintenance of outer membrane asymmetry in deep-rough mutants, considerable work must still be performed to distinguish between these models.

Other less drastic alterations in LPS composition, including the *lps*A, *gal*U, *rfa*Rd1, *rfa*D, *rfa*H, and *tol*D,E mutants of *E. coli* or *S. typhimurium,* and mutant AK43 of *P. aeruginosa,* cause supersusceptibility to hydrophobic antibiotics, detergents, and EDTA and, where studied, enhanced uptake of gentian violet (Table 1). Generally, the magnitude of these effects is related to the extent of the LPS alterations.

Other Mutational Alterations

Mutants deficient in Braun's liprotein *(lpo)* or in the covalently peptidoglycan-bound form of lipoprotein (*lky* D) in *E. coli* and *S. typhimurium* respectively, have conditional alterations in the permeability of the outer membrane to hydrophobic compounds (47, 176, 201, 213) and in the ability of the outer membrane to prevent leakage of periplasmic components (47, 213). The effects on permeability are reversible by Mg^{2+} (165, 213). The alteration in permeability may occur specifically at the site of the division septum formation, because the *lky*D mutation has been shown to cause blebbing of the outer membrane at this position (201).

A variety of colicin-tolerant mutants (cells able to bind specific colicins but not susceptible to their lethal action) in *E. coli* K-12 have been shown to be

more susceptible to hydrophobic antibiotics and detergents and, in some cases, are periplasmic-leaky (1, 10, 30, 41, 96, 109). One of these mutants, *tol* C, is thought to be altered in a minor outer-membrane protein [from gene-cloning experiments (P. Reeves, personal communication)]. Another mutant, *tol*A,B may be identical to the periplasmic leaky mutant *lky*::MuCts (1, 10, 30), which has been proposed to be altered in its LPS (1). Mapping and the phenotypes of another pair of periplasmic-leaky mutants *lky*-207 and *lky*-236 suggest that these too may be the same as the *tol* A,B mutations, although small differences exist (96).

In addition to the above specific examples, a variety of other mutants in outer membrane permeability to hydrophobic agents have been isolated and characterized (see Table 1). The types of permeability alterations in these are frequently similar, although minor differences exist in susceptibility to given agents. Some of these differences can be explained by different assay conditions; for example, Mg^{2+} is important (see Table 3), as is the concentration of the agent being tested. Taken overall these data argue for a multicomponent hydrophobic uptake system that can be "activated" by a variety of different mutational events. In addition to hypersensitivity to hydrophobic antibiotics, these mutants are usually somewhat more susceptible to EDTA and polycations.

ENHANCEMENT OF OUTER MEMBRANE PERMEABILITY BY CHEMICALS AND ANTIBIOTICS

EDTA

EDTA causes increased outer membrane permeability to many compounds in a wide range of gram-negative bacteria (Table 2). Central to the mode of action of EDTA is its strong divalent cation-chelating function (98), although it should be stressed that, with the exception of *P. aeruginosa* (59), an organic cation (usually Tris is used) and an appropriate mild alkaline pH are also required. The actual mechanism of outer membrane permeabilization is unknown. EDTA apparently causes the loss of substantial amounts of LPS [between 30% and 67% of the total in *E. coli* (60)], at either 37° C or 4° C (99), although the outer membrane apparently remains continuous after such treatment (54, 197), in that there do not appear to be easily observeable holes in the outer membrane.

The major alteration in outer-membrane substructure is in the reduction of the number of particles and pits upon freeze fracture (197). The LPS that is released can be separated into two fractions by ultracentrifugation (99). One, fraction F1, contains associated protein and lipid. The other, F2, which may have different sugar composition than bulk LPS, is apparently pure LPS. Data from mutant (198) and reconstitution (60) studies have suggested that it is the release of F2 that results in outer membrane permeabilization. For example,

Table 2 Outer membrane permeabilizing agents[a]

Agent	Bacteria[b]	Outer membrane permeabilization to[c]				Enhanced susceptibility to[d]		Other effects	References
		HPO	HPI	Lysozyme	PERI	HPO	HPI		
EDTA	many	1, 2, 3, 4	5, 6, 7	+	−	9, 10, 11, 12, 13, 14	18, 19, 20, 21, 22	A, B	5, 18, 25, 31, 59, 65, 69, 75, 81, 98, 108, 139, 155, 167, 174, 175, 185, 202
Ascorbate	P	1	5	+		9, 15	18, 19, 20, 21, 22		77, 136
Tris	E, P		5, 8	+	+			C	75, 77, 86, 185
Polymyxin B	many	2, 3, 4	5	+	+	10, 12, 13, 15	19, 20	C, D	22, 62, 77, 95, 112, 155, 172, 173, 183, 187
Deacylated polymyxin	E				−	9, 10, 11, 12	18	C	186, 188, 190
Octapeptin	E				+	13		A	185
Aminoglycosides	P	1, 2	5	+	−		18	A	75, 77, 100, 177
Polylysine	E, S, P	1	5	+	−	9, 11, 13	18	A	77
Benzalkonium chloride	E				+	13			185
Protamine	E				−	9, 11, 13		A	185, 188
BPI	E, S					10, 14		B	39, 203
Serum and/or complement	E			+	+		18, 19, 21, 22	D	31, 32, 35, 56, 211
Benzyl penicillin	P				+	13			114, 124
Ca^{2+} pretreatment	E	4	6	+	+	14, 17			13, 147, 155

[a]Abbreviations: BPI = bactericidal/permeability increasing protein; HPO = hydrophobic compounds; HPI = hydrophilic compounds; PERI = periplasmic enzymes—often β-lactamase or alkaline phosphatase.

[b]Bacterial species studied: E = *E. coli;* P = *P. aeruginosa;* S = *S. typhimurum.*

[c]The numbers signify the following compounds: 1 = N-phenyl napthylamine; 2 = 1-anilino-8-napthosulphonate; 3 = Gentian violet; 4 = dansyl chloride; 5 = nitrocefin; 6 = AMP or DNA; 7 = benzyl penicillin; 8 = paranitrophenyl phosphate; blank = not studied; + equals positive result; − equals no permeabilization to periplasmic enzymes observed.

[d]The numbers signify the following compounds: 9 = erythromycin; 10 = rifampicin; 11 = novobiocin; 12 = serum and/or complement; 13 = deoxycholate and/or cholate; 14 = actinomycin D; 15 = trimethoprim; 16 = bacitracin; 17 = nigericin, valinomycin, CCCP; 18 = β-lactam antibiotics; 19 = tetracyclines; 20 = chloramphenicol; 21 = aminoglycoside antibiotics; 22 = polymyxin B.

[e]The block capital letters mean: A = causes LPS release; B = enhances phospholipase sensitivity of cells; C = binds to LPS; D = causes outer membrane disorganization.

Table 3 Mg^{2+} reversal of the enhancement, by agents or mutants, of outer membrane permeability

Permeabilizing agent or mutant	Properties reversed by Mg^{2+}	References
Porin⁻ mutant	periplasmic β-lactamase leakage	11
Env mutant	Lysozyme sensitivity and autolysis	66
*tol*A,B mutant	Vancomycin sensitivity	10
*tol*D mutant	colicin tolerance; cell envelope stability; resistance to ampicillin; sensitivity to cholate	20, 123
Deep-rough (*rfa*) mutants	Hypersensitivity to Erythromycin, Bacitracin, Vancomycin, and Novobiocin; uptake of Gentian violet; leakage of periplasmic enzymes; outer membrane freeze-fracture morphology	85, 168
lpo mutant	Leakage of periplasmic β-lactamase; growth defect; outer membrane "blebbing"; EDTA hypersensitivity	7, 176, 213
Tris in growth medium	Release of alkaline phosphatase	24
Polymxin B	Killing; lytic activity, uptake of hydrophobic fluorescent compounds; formation of outer membrane blebs; release of periplasmic enzymes; enhanced nitrocefin uptake	22, 77, 112, 113, 153, 155
Octapeptin	Killing; phospholipid release	143
Aminoglycosides	Killing; uptake; permeability of outer membrane to nitrocefin, lysozyme, hydrophobic fluorescent compounds	19, 75, 100
Bactericidal/permeability increasing protein	Actinomycin D, rifampicin sensitivity	39, 203

EDTA-resistant mutants can be induced to release up to 30% of their LPS, primarily F1, without dramatic alterations in permeability (198).

In *P. aeruginosa*, a more substantial amount of protein is released during EDTA treatment. However, the outer membrane ultrastructural alterations are apparently similar to those of *E. coli* (54, 197).

Polymyxins

Polymyxin B (and octapeptin) causes rodlike projections (blebs) of the outer membranes of gram-negative bacteria (53, 154, 189). For this effect, and for a wide range of outer membrane permeabilization events mediated by polymyxin (Table 3), the fatty acyl tail is not required, because deacylated polymyxin [polymyxin B nonapeptide (189)] causes similar blebbling. Interestingly,

polymyxin B is different from EDTA in that it apparently releases little LPS into the medium (189). Because the polycationic part of polymyxin B is capable of displacing divalent cations by competition while EDTA removes divalent cations by chelation, it is tempting to speculate that these blebs represent an intermediate step in the release of LPS caused by EDTA treatment. In agreement with this proposal, not only do polymyxin B and EDTA cause similar permeabilization events, but outer membrane mutants exist in both *S. typhimurium (pmx)* and *P. aeruginosa* (PX^r) that are cross-resistant to both agents (see Table 1).

In any case, the blebbed outer membrane appears to be quite unstable, because polymyxin B–treated cells release substantial levels of periplasmic proteins (22) whereas EDTA-treated cells release only very small amounts of these proteins. Other polycations, like protamine and polylysine, are more like EDTA in their outer membrane effects because they cause substantial LPS release but no periplasmic protein release and little or no outer membrane blebbing (189).

The release of periplasmic proteins by polymyxin B, but not other outer membrane permeability effects, may be a function of possession of a hydrophobic tail (189). Model studies have suggested a possible mechanism for this polymyxin B effect, because it interacts with both the polar and nonpolar regions of lipids (131) to destabilize bilayers (106). This explanation, however, is probably an oversimplification, because it does not explain the similarities between the effects of polymyxins and EDTA. Polymyxin B is known to interact with the divalent-cation binding sites on LPS (74, 153), and such binding is almost certainly critical to its permeabilizing function, because polymyxin effects are blocked by Mg^{2+} (113).

Aminoglycosides

Polycationic aminoglycoside antibiotics (e.g., streptomycin, gentamicin) interact with divalent-cation binding sites on *P. aeruginosa* LPS (E. McGroarty, unpublished) and permeabilize the outer membranes of this organism (75, 100). A number of lines of evidence suggest that aminoglycosides act at the same site as do polymyxins and EDTA (70, 74, 113). We have proposed that the interaction of aminoglycosides with *P. aeruginosa* outer membranes at this site is part of an uptake mechanism (i.e. self-promoted uptake across the outer membrane of this organism) (see above).

Tris

The large organic cation, Tris, is not only capable of assisting the action of EDTA (59) but is also able to permeabilize outer membranes itself, when used in moderately high concentrations (75, 86). This effect is probably related to its ability to interact with LPS, albeit with lower affinity than e.g. polymyxin

(154). The results of Voss (199) are suggestive that this ability to permeabilize outer membranes may also be shared by other organic cations.

Ca^{2+} Pretreatment and DNA Uptake

The development of competence [i.e. the ability of a cell to be genetically transformed by exogenous DNA] usually involves two stages: (*a*) incubation of a concentrated cell suspension at O°C, in the presence of exogenous DNA, in buffer containing 25–100 mM $CaCl_2$; and (*b*) a brief (6–15 min) temperature shock caused by raising the temprature of the buffer (147, 180, 196). The temperature shock seems to be dispensible in some species of bacteria, and individual researchers use somewhat different procedures.

However, the net result of this procedure is to permeabilize the outer membrane to a variety of hydrophobic and hydrophilic compounds (Table 2). This treatment also allows vectorial transport (i.e. uptake) of macromolecules, including large DNA molecules (63), antibodies, amylopectin, and periplasmic maltose and galactose-binding proteins (13) across the outer membrane. However, development of competence does not cause large holes in the outer membrane (63, 87) and does not permit significant leakage of periplasmic enzymes into the environment (13).

The uptake of DNA across the outer membrane probably involves an LPS site, because rough mutants of *E. coli* are more easily transformed than smooth (O-antigen-containing) strains (179, 180). In addition, there is substantial variation in the competence of different isogenic rough LPS mutants (179). The involvement of divalent cations [usually Ca^{2+}, although Mg^{2+} and Ba^{2+} also work (33, 179, 204)] in the induction of competence may indicate that the same LPS divalent cation–crossbridging sites discussed above are involved. In agreement with this, Tris-EDTA-lysozyme–induced spheroplasts will also take up DNA, providing an appropriate polycationic compound is present (82).

The mechanism of DNA uptake is not well understood, but it has been postulated to involve the formation of transient channels through the membrane. These channels are proposed to be open only during the interaction of the nucleic acid with the membrane, and after nucleic acid uptake is finished the channel is destroyed or closed (63). [*Note:* Although this proposal was made for DNA uptake across the inner membrane, it seems equally valid for outer-membrane uptake that apparently occurs with higher efficiency (204).] Van Die et al (196) have suggested that a phase transition (caused by the temperature shock) and/or the formation of nonbilayer structures (caused by Ca^{2+}) may be important in formation of the temporary channels.

On the other hand, Grinius (63) suggests that Ca^{2+} binds and confers a net positive charge on DNA, converting it to a polycation. In agreement with this, divalent cations promote the binding of DNA to *E. coli* and *H. influenza* outer membranes (33, 204). Interestingly, this hypothesis would imply that DNA might interact with cells in ways similar to other polycations. Kahn et al (87) have shown that competent *Haemophilus* cells produce outer membrane blebs reminiscent of those that appear due to polymyxin B and deacylated polymyxin B treatment of *E. coli* (189).

Serum and/or Complement

The bactericidal killing of gram-negative cells by serum usually involves one of two complement cascade pathways (107). Each of these pathways is activated in a unique fashion, although the final steps and proteins involved are common. The effect of at least one complement component is to disrupt the outer membrane, as judged by not only electron microscopic observation (207) but also enhanced uptake of lysozyme (56, 207) and release of periplasmic enzymes (211) by complement-treated cells. The permeabilization event has been proposed (211) to be important for allowing passage through the outer membrane and subsequent incorporation into the inner membrane of the channel-forming component C9.

The mechanism of complement permeabilization of outer membranes may well be similar to the self-promoted uptake mechanism discussed above. Evidence for this includes the synergy of aminoglycosides, polymyxins, and other polycationic compounds with serum and/or complement (31, 32, 43) as well as the binding of complement to the lipid A portion of LPS (50, 208) and the inhibition of this binding by polmyxin B or Mg^{2+} or Ca^{2+} (suggesting competition for an LPS site) (49, 208). In addition, rough LPS-altered organisms are more sensitive to serum bactericidal (complement) killing than smooth cells (145), a result usually taken to reflect differences in the carboyhydrate portion of LPS, but which may instead reflect the differential affinities of smooth and rough LPS for complement and Mg^{2+} (49).

Bactericidal/Permeability–Increasing Protein from Polymorphonuclear Leukocytes

A protein from polymorphonuclear leukocytes with an isoelectric point of 9.5–10.0 kills several species of gram-negative bacteria (203). Rough strains are more susceptible than smooth strains and, like polymyxin, this highly cationic protein (called bactericidal/permeability–increasing protein) is quite inactive against gram-positive cells (39). In addition, Mg^{2+}-limited cells of *Pseudomonas aeruginosa*, which have been shown to be resistant to polymyxin B, aminoglycosides, and EDTA (113; see PX^r in Table 1), are resistant to the action of this protein (44). The protein results in permeabilization of outer membranes to actinomycin D and rifampicin, and the permeabilization event is antagonized by either Mg^{2+} or Ca^{2+} (39, 203). Permeability induction requires the continuous presence of bound bactericidal/permeability–increasing protein, in addition to at least two postbinding steps. The proposed mechanism of interaction of this cationic protein bears many similarities to the self-promoted uptake mechanism (203).

ENERGIZATION STATE AND HYDROPHOBIC PERMEABILITY

A number of authors have demonstrated that de-energization of cells in a variety of ways results in increased fluorescence of hydrophobic fluorescent probes added to the cell suspension (80). This is true of negatively charged (81, 116, 181), positively charged (15), or neutral (81, 166) probes added to cells de-energized in a variety of ways. Membrane de-energization will increase the fluorescence of exogenously added cis-parinaric acid but not biosynthetically incorporated, esterified parinaric acid (181). This data, together with direct measurement of N-phenyl napthylamine (NPN) disappearance from the environment, led Helgerson & Cramer (80, 81) to propose that de-energization resulted in an alteration in outer membrane permeability to the probes; that is, the enhancement was due to partitioning of the probes into the more hydrophobic membrane interiors.

In agreement with this it could be shown that EDTA (81), polymyxin B (112), and aminoglycosides (100) cause similar uptake of hydrophobic fluorescent probes. Also, added Mg^{2+} decreased the rate of fluroescence increase due to polymyxin B (112), aminoglycosides (100), and two colicins that are presumed to cause alterations in membrane energization (15, 184). In addition, deep-rough LPS-altered mutants of *Salmonella,* which exhibit many other outer membrane permeability alterations (see Table 1), showed no enhanced uptake of the hydrophobic, photolabile probe azidopyrene upon de-energization, in marked contrast to wild-type *Salmonella* strains, which showed a 2–5-fold enhancement of uptake (209; Wolf & Konisky, submitted). This data implies that either de-energization of cells results in a marked structural change in the outer membrane, or there is an energized secretion mechanism for hydrophobic compounds in untreated wild-type cells.

In favor of the latter view, re-energization of cells causes rapid loss of NPN from the cells (80, 81). In addition, energy metabolism is required to reverse EDTA-mediated outer membrane permeability alterations (45). In support of the former idea, de-energization of cells by CCCP results in enhanced rates of DNA uptake into Ca^{2+}-treated cells in the absence of a temperature shock (146), prevents irreversible binding of bacteriophages T1 and 80 to their outer membrane receptors (71), and blocks a variety of *ton* B–protein–mediated transport systems (73), possibly at the level of the outer membrane.

PHYSICAL DAMAGE OF OUTER MEMBRANES

Outer membranes can also be damaged by physical treatments. For example, *E. coli* cells exposed to their aquatic or reagent grade water become susceptible to deoxycholate and lysozyme (215). Electron microscopic examination of

such cells showed outer membrane blebbing. Similarly, aerosolization of *E. coli* makes the outer membrane permeable to lysozyme (68). The sensitivity of aerosolized *E. coli* to lysozyme can be partly reversed by Mg^{2+}, although as with EDTA treatment of cells, full repair of outer membranes required energy metabolism.

Freeze drying of *Salmonella anatum* results in enhanced susceptibility of cells to EDTA, actinomycin D, penicillin, tetracycline, and chloramphenicol, suggesting outer-membrane damage (138). Once again energy metabolism is required to repair outer membrane damage. Similarly, freeze-thawing *E. coli* strains results in release of cyclic phosphodiesterase (21) as well as enhanced susceptibility to dyes, enzymes, and detergents (6), and loss of LPS. The effects of freeze-thawing on outer membrane permeability are more extreme in deep-rough mutants than in smooth strains (6).

GROWTH AND MEDIA EFFECTS

A number of studies have examined the influence of growth in various media on susceptibility to antibiotics, but only rarely have outer membrane permeability alterations been definitively demonstrated. Perhaps the best example is *P. aeruginosa* cells grown in Mg^{2+}-limited medium, which are phenotypically indistinguishable from the genetically polymyxin-, aminoglycoside-, EDTA-resistant mutants (PX^r) described in Table 1. Another example is *P. fluorescens* cells grown on succinate, which demonstrate higher sensitivity to actinomycin D and to the permeabilizing effects of EDTA than the same cells grown on glucose (200). In addition, phosphate limitation of *P. fluorescens* renders these cells polymyxin B–resistant and decreases the capacity of outer membranes to bind polymyxin B (36).

EXCRETION OF PROTEINS ACROSS THE OUTER MEMBRANE

Whereas some gram-negative bacteria (e.g. *E. coli*) have few, if any, proteins that are excreted out of the cell, others like *P. aeruginosa* are known to excrete a range of polypeptides (55, 170). The secretion of proteins across the outer membrane may well relate to the specific nature of the organism and its outer membrane. The excretion by *P. aeruginosa* of phospholipase C (and another enzyme, alkaline phosphatase, which is often used in other bacteria as a periplasmic marker enzyme) is specific, because it has been shown that its excretion is not accompanied by the excretion of periplasmic β-lactamase or phosphate-binding protein (135).

In addition, during phospholipase secretion, outer membrane permeability to the β-lactam nitrocefin is unaltered (135). Despite this, the levels of alkaline

phosphatase and phospholipase C increase in both the periplasm and the external medium after phosphate limitation. Thus it seems that either a specific alteration in outer membrane permeability has occurred, or these enzymes are secreted to the outer membrane and from there released either inwards (to the periplasm) or outwards (to the external medium).

DIVALENT CATION REVERSAL OF OUTER MEMBRANE PERMEABILITY ALTERATIONS

One of the most interesting aspects of the alteration of outer membrane permeability by mutants and by various agents is that in those instances where it has been examined, exogenously added Mg^{2+} reverses the permeability changes (Table 3). This is true of even the classical outer-membrane permeabilizing agents and mutants (e.g. deep-rough mutants, polymyxin B, EDTA). This provides strong evidence that a single class of outer membrane site is involved in all of these permeability alterations, presumably a Mg^{2+}-binding site. As clearly demonstrated by Schindler & Osborn (153) and by McGroarty and coworkers (27), LPS has a strong divalent cation–binding site.

A HYPOTHESIS TO EXPLAIN OUTER MEMBRANE PERMEABILITY ALTERATIONS

It is my belief that the majority of the outer membrane permeability alterations discussed in this review can be simply explained by postulating that they involve sites at which divalent cations noncovalently cross-bridge adjacent LPS molecules. This proposal is not unique and has been made for a number of the pemeabilization events discussed; however, the universality of the proposal has not been widely recognized. The evidence suggesting a single class of outer membrane sites involved in permeability alterations includes (*a*) the chemical similarities of permeabilizing agents (usually polycations, organic cations, or divalent cation chelators—Table 2), (*b*) the antagonism of agents and mutants by Mg^{2+} (Table 3), (*c*) the similar types of lesions caused by most mutants and agents (Tables 1 and 2) (with destruction of the permeability barrier to hydrophobic compounds featuring prominently), and (*d*) the occurence of outer membrane mutants altered in their susceptibility to many outer-membrane permeabilizing agents (especially PX^r and *pmr*A in Table 1).

The differences in permeability alterations between individual mutations could be explained on the basis of the specific nature of the mutants, which are largely unknown. Similarly the differences in alterations of outer-membrane permeability caused by different agents could be explained by competition for the permeabilized site between the permeabilizing agent and the compounds being studied. With this in mind it should be noted that two distinct permeabilization events to hydrophobic agents and detergents like sodium dodecyl sulphate have been proposed (189).

Whereas the site of outer membrane permeabilization can be predicted, little is known or understood about the specific nature of the underlying lesions. Despite the secretion of copious amounts of LPS from EDTA-treated cells, the outer membrane remains continuous across the cell surface (54, 197), as indeed it does with other outer membrane permeabilizing agents (53, 63, 86, 90, 97, 153, 189) or for various hyperpermeable mutants (45, 85, 201). Therefore, it does not seem likely that the presence of holes through the outer membrane can explain the permeabilization (the specificity of the lesions also favors this argument). Nor does lipid flip-flop giving rise to areas of phospholipid bilayer in the outer membrane [as suggested for deep-rough mutants (164)] provide an easy explanation for these lesions, because it is difficult to understand how a lipid bilayer allows the enhanced passage of hydrophobic compounds and lysozyme (or DNA) as seen for many of the permeability alterations.

Although any explanation of the mechanism whereby compounds pass across permeabilized outer membranes is premature, a number of possibilities exist. For example, the removal or displacement of divalent cations from their LPS binding sites may cause a structural discontinuity, such as nonbilayer structures (28), membrane blebs (53, 153), elastic distortion of the bilayer (79), or regions of enhanced fluidity (144) in the outer membrane. The interaction of molecules with the surface charges surrounding such structures and partitioning into the hydrophobic regions of the structure may explain the permeability to both specific hydrophilic compounds and hydrophobic substances respectively. This proposal is deliberately vague and poses more questions than it answers. For example, (*a*) What is the nature of the discontinuity? (*b*) What is the role of particles and pits (102, 197) in the outer membrane interior? (*c*) Why do only some permeabilizing agents cause blebs? (*d*) What is the role of energization in preventing such structural discontinuities from forming?

The physiological importance of LPS divalent cation crossbridges (for outer membrane stability and as a component of the hydrophobic barrier) is clear. In contrast, the physiological significance of self-promoted uptake, aside from a possible role in transformation, is arguable. However, the existence of such an LPS site may have strong medical relevance, because it constitutes an "Achille's heel" for the cell. (This site is apparently attacked by complement, polymyxins, aminoglycosides (in *P. aeruginosa),* and cationic proteins from polymorphonuclear leukocytes). Given the strong influence of the outer membrane permeability barrier on rate of uptake and the susceptibility of gram-negative bacteria to antibiotics (2, 117, 119), agents that enhance outer membrane permeability should prove synergistic with antibiotics. This has been frequently demonstrated in vitro (see Table 2), while clinical trials with EDTA (206) and ascorbate (137) have been quite promising.

Thus, future studies on agents capable of enhancing outer-membrane permeability should be directed towards understanding the mechanism of permeabilization as well as searching for medically useful outer-membrane permeability–altering compounds.

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Literature Cited

1. Anderson, J. J., Wilson, J. M., Oxender, D. L. 1979. *J. Bacteriol.* 140:351–58
2. Angus, B. L., Carey, A. M., Caron, D. A., Kropinski, A. M. B., Hancock, R. E. W. 1982. *Antimicrob. Agents Chemother.* 21:299–309
3. Bavoil, P., Nikaido, H., von Meyenburg, K. 1977. *Mol. Gen. Genet.* 158: 23–33
4. Beacham, I. R., Haas, P., Yagil, E. 1977. *J. Bacteriol.* 129:1034–44
5. Beacham, I. R., Kahana, R., Levy, L., Yagil, E. 1973. *J. Bacteriol.* 116:957–64
6. Bennet, G. M., Seaver, A., Calcott, P. H. 1981. *Appl. Environ. Microbiol.* 42:843–49
7. Benz, R., Boehler-Kohler, B. A., Dieterle, R., Boos, W. 1978. *J. Bacteriol.* 135:1080–90
8. Benz, R., Hancock, R. E. W. 1981. *Biochim. Biophys. Acta* 646:298–308
9. Benz, R., Hancock, R. E. W., Nakae, T. 1982. *Transport in Biomembranes: Model Systems and Reconstitution.* pp. 123–34. New York: Raven 272 pp.
10. Bernstein, A., Rolfe, B., Onodera, K. 1972. *J. Bacteriol.* 112:74–83
11. Boehler-Kohler, B. A., Boos, W., Dieterle, R., Benz, R. 1979. *J. Bacteriol.* 138:33–39
12. Branes, L. V., Kay, W. W. 1983. *J. Bacteriol.* 154:1462–66
13. Brass, J. M., Boos, W., Hengge, R. 1981. *J. Bacteriol.* 146:10–17
14. Braun, V., Sieglun, U. 1970. *Eur. J. Biochem.* 13:336–46
15. Brewer, G. J. 1976. *Biochemistry* 15: 1387–92
16. Brinton, C. C., Buzzell, Z., Lauffer, M. A. 1964. *Biochim. Biophys. Acta* 15: 533–40
17. Brown, M. R. W. 1975. *Resistance of Pseudomonas Aeruginosa,* pp. 71–107. London: Wiley
18. Brown, M. R. W., Richards, R. M. E., 1965. *Nature* 207:1391–93
19. Bryan, L. E., van den Elzen, H. M. 1977. *Antimicrob. Agents Chemother.* 12:163–77
20. Burman, L. G., Nordstrom, K. 1971. *J. Bacteriol.* 106:1–13
21. Calcott, P. H., McLeod, R. A. 1975. *Can. J. Microbiol.* 21:1724–32
22. Gerny, G., Teuber, M. 1971. *Arch. Microbiol.* 78:166–79
23. Champlin, F. R., Gilleland, M. E., Conrad, R. S. 1983. *Antimicrob. Agents Chemother.* 24:5–9
24. Cheng, K.-J., Ingram, J. M., Costerton, J. W. 1970. *J. Bacteriol.* 104:748–43
25. Cleeland, R., Beskid, G., Grunberg, E. 1970. *Infect. Immun.* 2:371–75
26. Coleman, W. G., Leive, L. 1979. *J. Bacteriol.* 139:899–910
27. Coughlin, R. T., Caldwell, C. R., Haug, A., McGroarty, E. J. 1981. *Biochem. Biophys. Res. Commun.* 100:1137–42
28. Cullis, P. R., de Kruijff, B. 1979. *Biochim. Biophys. Acta* 559:399–420
29. Dame, J. B., Shapiro, B. M. 1976. *J. Bacteriol.* 127:961–72
30. Davies, J. K., Reeves, P. 1975. *J. Bacteriol.* 123:102–17
31. Davis, S. D., Iannett, A. 1972. *Appl. Microbiol.* 23:775–79
32. Davis, S. D., Iannett, A. 1978. *Antimicrob. Agents Chemother.* 13:820–25
33. Deich, R. A., Hoyer, L. C. 1982. *J. Bacteriol.* 152:855–64
34. Dirienzo, J. M., Nakamura, K., Inouye, M. 1978. *Ann. Rev. Biochem.* 47:481–532
35. Donaldson, D. M., Roberts, R. R., Larsen, H. S., Tew, J. G. 1974. *Infect. Immun.* 10:657–66
36. Dorrer, E., Teuber, M. 1977. *Arch. Microbiol.* 114:87–89
37. Douglas, J., Lee, M. D., Nikaido, H. 1981. *FEMS Microbiol. Lett.* 12:305–9
38. Egan, A. F., Russell, R. R. B. 1973. *Genet. Res.* 21:139–52
39. Elsbach, P., Weiss, J., Franson, R. C., Beckerdite-Quagliata, S., Schneider, A., Harris, L. 1979. *J. Biol. Chem.* 254:11000–9
40. Ennis, H. L. 1971. *J. Bacteriol.* 107: 486–90
41. Eriksson-Grennberg, K. G., Nordstrum, K. 1973. *J. Bacteriol.* 115:1219–22
42. Fass, R. J., Barnisham, J. 1980. *Rev. Infect. Dis.* 2:841–53
43. Fierer, J., Finley, F. 1979. *J. Infect. Dis.* 140:581–89
44. Finch, J. E., Brown, M. R. W. 1978. *Infect. Immun.* 20:340–46
45. Foulds, J. 1976. *J. Bacteriol.* 128:604–8
46. Franklin, T. J., Snow, G. A. 1981. *Biochemistry of Antimicrobial Action.* London: Chapman & Hall. 217 pp. 3rd Ed.
47. Fung, J., MacAlister, T. J., Rothfield, L. I. 1978. *J. Bacteriol.* 133:1467–71
48. Galanos, C., Luderitz, O., Rietschel, E. T., Westphal, O. 1977. *Int. Rev. Biochem.* 14:239–335
49. Galanos, C., Rietschel, E. T., Luderitz, O., Westphal, O. *Eur. J. Biochem.* 19: 143–52
50. Gewurz, H., Shin, H. S., Mergenhagen, S. E. 1968. *J. Exp. Med.* 128:1049–56
51. Gilleland, H. E., Conrad, R. S. 1982. *Antimicrob. Agents Chemother.* 22: 1012–16
52. Gilleland, H. E., Farley, L. B. 1982. *Can. J. Microbiol.* 28:830–40
53. Gilleland, H. E., Murray, R. G. E. 1976. *J. Bacteriol.* 125:267–81
54. Gilleland, H. E., Stinnett, J. D., Eagon, R. G. 1974. *J. Bacteriol.* 117:302–11
55. Glenn, A. R. 1976. *Ann. Rev. Microbiol.* 30:41–62
56. Glynn, A. A. 1969. *Immunology* 16: 463–68
57. Gmeiner, J., Schlecht, S. 1979. *Eur. J. Biochem.* 93:609–20
58. Gmeiner, J., Schlecht, S. 1980. *Arch. Microbiol.* 127:81–86
59. Goldschmidt, M. C., Wyss, O. 1967. *J. Gen. Microbiol.* 47:421–31
60. Graham, G. S., Treick, R. W., Brunner, D. P. 1979. *Curr. Microbiol.* 2:339–43
61. Gray, G. W., Wilkinson, S. G. 1965. *J. Gen. Microbiol.* 39:385–93
62. Greenfield, S., Feingold, D. S. 1970. *J. Infect. Dis.* 121:555–58
63. Grinius, L. 1980. *FEBS Lett.* 113:1–10
64. Grundstrom, T., Normark, S., Magnusson, K.-E. 1980. *J. Bacteriol.* 144:884–90
65. Gustafsson, P., Nordstrom, K., Normark, S. 1973. *J. Bacteriol.* 116:893–900
66. Guymon, L. F., Sparlin, P. F. 1975. *J. Bacteriol.* 124:757–63

67. Guymon, L. F., Walstad, D. L., Sparling, P. F. 1978. *J. Bacteriol.* 136:391–401
68. Hambleton, P. 1971. *J. Gen. Microbiol.* 69:81–88
69. Hamilton-Miller, J. M. T. 1965. *Biochem. Biophys. Res. Commun.* 20: 688–91
70. Hancock, R. E. W. 1981. *J. Antimicrob. Chemother.* 8:429–45
71. Hancock, R. E. W., Braun, V. 1976. *J. Bacteriol.* 125:409–15
72. Hancock, R. E. W., Decad, G. M., Nikaido, H. 1979. *Biochem. Biophys. Acta* 554:323–29
73. Hancock, R. E. W., Hantke, K., Braun, V. 1977. *Arch. Microbiol.* 114:231–39
74. Hancock, R. E. W., Nicas, T. I. 1984. *Antimicrobial Drug Resistance,* in press. New York: Academic 999 pp.
75. Hancock, R. E. W., Raffle, V. J., Nicas, T. I. 1981. *Antimicrob. Agents Chemother.* 19:777–85
76. Hancock, R. E. W., Reeves, P. 1976. *J. Bacteriol.* 127:98–108
77. Hancock, R. E. W., Wong, P. 1984. *Antimicrob. Agents Chemother.* In press
78. Harder, K. J., Nikaido, H., Mitsuhashi, M. 1981. *Antimicrob. Agents Chemother.* 20:549–52
79. Hartmann, W., Galla, H., Sackmann, E. 1978. *Biochim. Biophys. Acta* 510:124–39
80. Helgerson, S. L., Cramer, W. A. 1976. *J. Supramol. Struct.* 5:291–308
81. Helgerson, S. L., Cramer, W. A. 1977. *Biochemistry* 16:4109–17
82. Henner, W. D., Kleber, I., Benzinger, R. 1973. *J. Virol.* 12:741–47
83. Henning, Y., Haller, I. 1975. *FEBS Lett.* 55:161–64
84. Inouye, M., ed. 1979. *Bacterial Outer Membranes—Biogenesis and Functions.* New York: Wiley 534 pp.
85. Irvin, R. T., Chatterjee, A. K., Sanderson, K. E., Costerton, J. W. 1975. *J. Bacteriol.* 124:930–41
86. Irvin, R. T., MacAlister, T. J., Costerton, J. W. 1981. *J. Bacteriol.* 145:1397–403
87. Jaffe, A., Chabbert, Y. A., Semonin, O. 1982. *Antimicrob. Agents Chemother.* 22:942–48
87. Kahn, M. E., Maul, G., Goodgal, S. M. 1982. *Proc. Natl. Acad. Sci. USA* 79: 6370–74
88. Kamio, Y., Nikaido, H. 1976. *Biochemistry* 15:2561–70
89. Kennedy, E. P. 1982. *Proc. Natl. Acad. Sci. USA* 79:1092–95
90. Koike, M., Iida, K., Matsuo, M. 1969. *J. Bacteriol.* 97:448–52

91. Kropinski, Y., Murakami, K., Nishikawa, T. 1981. *Antimicrob. Agents Chemother.* 20:613–19
92. Kropinski, A. M. B., Chan, L., Milazzo, F. H. 1978. *Antimicrob. Agents Chemother.* 13:494–99
93. Kropinski, A. M., Kuzio, J., Angus, B. L., Hancock, R. E. W. 1982. *Antimicrob. Agents Chemother.* 21:310–19
94. Kusano, T., Izaki, K., Takahashi, H. 1975. *J. Antibiot.* 28:689–95
95. La Porte, D. C., Rosenthal, K. S., Storm, D. R. 1977. *Biochemistry* 16: 1642–48
96. Lazzaroni, J.-C., Portalier, R. C. 1981. *J. Bacteriol.* 145:1357–58
97. Leive, L. 1968. *J. Biol. Chem.* 243: 2373–80
98. Leive, L. 1974. *Ann. NY Acad. Sci.* 235:109–29
99. Leive, L., Shovlin, V. K., Mergenhagen, S. E. 1968. *J. Biol. Chem.* 243: 6384–91
100. Loh, B., Grant, C., Hancock, R. E. W. 1984. *Antimicrob. Agents Chemother.* In press
101. Lugtenberg, B. 1981. *Trends Biochem. Sci.* 6:262–66
102. Lugtenberg, B., van Alphen, L. 1983. *Biochim. Biophys. Acta* 737:51–115
103. Luktenhaus, J. F. 1977. *J. Bacteriol.* 131:631–37
104. Maier, T. W., Zubrzycki, L., Coyle, M. B., Chila, M., Warner, P. 1975. *J. Bacteriol.* 124:834–42
105. Makela, P. H., Sarvas, M., Calcagno, S., Lounatmaa, K. 1978. *FEMS Microbiol. Lett.* 3:323–26
106. Miller, I. R., Bach, D., Teuber, M., 1978. *J. Membr. Biol.* 39:49–56
107. Muller-Eberhard, H. J. 1975. *Ann. Rev. Biochem.* 44:697–724
108. Muschel, L. H., Gustafson, L. 1968. *J. Bacteriol.* 95:2010–13
109. Nagel de Zwaig, R., Luria, S. E. 1967. *J. Bacteriol.* 94:1112–23
110. Nakae, R., Nakae, T. 1982. *Antimicrob. Agents Chemother.* 22:554–59
111. Nakamura, H., Tojo, T., Greenberg, J. 1975. *J. Bacteriol.* 122:874–79
112. Newton, B. A. 1954. *J. Gen. Microbiol.* 10:491–99
112a. Nicas, T. I. 1983. *Ph.D. thesis.* Univ. British Columbia, Canada
113. Nicas, T. I., Hancock, R. E. W. 1980. *J. Bacteriol.* 143:872–78
114. Nicas, T. I., Hancock, R. E. W. 1983. *J. Bacteriol.* 153:281–85
115. Nicas, T. I., Hancock, R. E. W. 1983. *J. Gen. Microbiol.* 129:509–17
116. Nieva-Gomez, D., Gennis, R. B. 1977. *Proc. Natl. Acad. Sci. USA* 74:1811–15
117. Nikaido, H. 1976. *Biochim. Biophys. Acta* 433:118–32
118. Nikaido, H., Bavoil, P., Hirota, Y. 1977. *J. Bacteriol.* 132:1045–47
119. Nikaido, H., Nakae, T. 1979. *Adv. Microbiol. Physiol.* 21:163–250
120. Nikaido, H., Rosenberg, E. Y. 1983. *J. Bacteriol.* 153:241–52
121. Nikaido, H. Rosenberg, E. Y., Foulds, J. 1983. *J. Bacteriol.* 153:232–40
122. Nikaido, H., Song, S. A., Shaltiel, L., Nurminen, S. 1977. *Biochem. Biophys. Res. Commun.* 76:324–30
123. Nordstrom, K., Burman, L. G., Eriksson-Grennberg, K. G. 1970. *J. Bacteriol.* 101:659–68
124. Nordstrom, K., Sykes, R. B. 1974. *Antimicrob. Agents Chemother.* 6:741–46
125. Normark, S. 1969. *J. Bacteriol.* 98: 1274–77
126. Normark, S. 1970. *Genet. Res.* 16:63–78
127. Normark, S., Westling, B. 1971. *J. Bacteriol.* 108:45–50
128. Ohmori, H., Azuma, A., Suzuki, Y., Hashimoto, Y. 1977. *Antimicrob. Agents Chemother.* 12:537–39
129. Osborn, M. H., Wu, H. C. P. 1980. *Ann. Rev. Microbiol.* 34:369–422
130. Oberbeeke, N., Lugtenberg, B. 1982. *Eur. J. Biochem.* 126:113–18
131. Pache, W., Chapman, D., Hillaby, R. 1972. *Biochim. Biophys. Acta* 255:358–64
132. Palva, E. T., Makela, P. H. 1980. *Eur. J. Biochem.* 107:137–43
133. Payne, J. W., Gilvarg, C. 1968. *J. Biol. Chem.* 243:6291–301
134. Pazoles, C. J., Kulpa, C. F. 1977. *Biochim. Biophys. Acta* 466(1):160–75
135. Poole, K., Hancock, R. E. W. 1983. *FEMS Microbiol. Lett.* 16:25–29
135a. Raetz, C. R., Foulds, J. 1977. *J. Biol. Chem.* 252:5911–15
136. Rawal, B. D. 1978. *Chemotherapy* 24: 166–71
137. Rawal, B. D., McKay, G., Blackhall, M. I. 1974. *Med. J. Aust.* 1:169–74
138. Ray, B., Jezeski, J. J., Busta, F. F. 1971. *Appl. Microbiol.* 22:401–7
139. Reid, P., Speyer, J. 1970. *J. Bacteriol.* 104:376–86
140. Richmond, M. H., Clark, D. C., Wotton, S. 1979. *Antimicrob. Agents Chemother.* 10:215–18
141. Richmond, M. H., Sykes, R. B. 1973. *Adv. Microbiol. Physiol.* 9:31–86
142. Roantree, R. J., Kuo, T. T., MacPhee, D. G. 1977. *J. Gen. Microbiol.* 103:223–24
143. Rosenthal, K. S., Swanson, P. E., Storm, D. R. 1976. *Biochemistry* 15: 5783–92

144. Rotten, S., Leive, L. 1977. *J. Biol. Chem.* 252:2077–81
145. Rowley, D. 1968. *J. Bacteriol.* 95:1647–52
146. Sabelnikov, A. G., Domardsky, I. V. 1979. *Mol. Gen. Genet.* 172:313–17
147. Sabelnikov, A. G., Domaradsky, I. V. 1981. *J. Bacteriol.* 146:435–43
148. Saier, M. H. 1979. *The Bacteria,* VII:168–227. New York: Academic.
149. Sanderson, K. E., MacAlister, T., Costerton, J. W., Cheng, K. J. 1974. *Can. J. Microbiol.* 20:1135–45
150. Sanderson, K. E., Stocker, B. A. D. 1981. *J. Bacteriol.* 146:535–41
151. Sarubbi, F. A., Sparling, P. F., Blackman, E., Lewis, E. 1975. *J. Bacteriol.* 124:750–56
152. Sawai, T., Hiruma, R., Kawana, N., Kaneko, M., Taniyasu, F., et al. 1982. *Antimicrob. Agents Chemother.* 22:585–92
153. Schindler, M., Osborn, M. J., 1979. *Biochemistry* 18:4425–30
154. Schindler, P. G., Teuber, M. 1975. *Antimicrob. Agents Chemother.* 8:95–104
155. Schindler, P. R. G., Teuber, M. 1978. *J. Bacteriol.* 135:198–206
156. Schlecht, S., Schmidt, G. 1970. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig.* 212:505–511
157. Schlecht, S., Westphal, O. 1970. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig.* 213:356–81
158. Schmidt, G., Schlecth, S., Westphal, O. 1969. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig.* 212:88–96
159. Schweizer, M., Hindennach, I., Garten, W., Henning, U. 1978. *Eur. J. Biochem.* 82:211–17
160. Sha'afi, R. I., Gary-Bobo, C. M., Solomon, A. K. 1971. *J. Gen. Physiol.* 58:238–58
161. Shales, S., Chopra, I. 1982. *J. Antimicrob. Chemother.* 9:325–31
162. Sherbert, G. V., Lakshmi, M. S. 1973. *Biochim. Biophys. Acta* 298:50–58
163. Singh, A. P., Reithmeier, R. A. F. 1975. *J. Gen. Appl. Microb.* 21:109–18
164. Smit, J., Kanno, Y., Nikaido, H. 1975. *J. Bacteriol.* 124:942–58
165. Sonntag, I., Schwarz, H., Hirota, Y., Henning, U. 1978. *J. Bacteriol.* 136: 280–85
166. Sparling, P. F., Sarrubi, F. A., Blackman, E. 1975. *J. Bacteriol.* 124:740–49
167. Spicer, A. B., Spooner, D. F. 1974. *J. Gen. Microbiol.* 80:37–50
168. Stan Lotter, H., Gupta, M., Sanderson, K. E. 1979. *Can. J. Microbiol.* 25:475–85
169. Stein, W. D. 1967. *The Movement of Molecules Across Cell Membranes.* New York: Academic
170. Stinson, M. W., Hayden, C. 1979. *Infect. Immun.* 25:558–64
171. Stock, J. B., Rauch, B., Roseman, S. 1977. *J. Biol. Chem.* 252:7850–61
172. Storm, D. R., Rosenthal, K. S., Swanson, P. E. 1977. *Ann. Rev. Biochm.* 46:723–63
173. Sud, I. J., Feingold, D. S. 1975. *Antimicrob. Agents Chemother.* 8:34–37
174. Suginaka, H., Kotani, S., Takata, N., Ogawa, M. 1980. *FEMS Microbiol. Lett.* 8:79–82
175. Suginaka, H., Schimatani, M., Kotani, S., Ogawa, M., Hama, M., et al. 1979. *FEMS Microbiol. Lett.* 5:177–79
176. Suzuki, H., Nishimura, Y., Yasuda, S., Nishimura, A., Yamada, M., et al. 1978. *Molec. Gen. Genet.* 167:1–9
177. Sykes, R., Morris, A. 1975. *Prog. Med. Chem.* 12:333–93
178. Tamaki, S., Sato, T., Matsuhashi, M. 1971. *J. Bacteriol.* 105:968–75
179. Taketo, A. 1977. *Z. Naturforsch.* 32c:429–33
180. Taketo, A. 1978. *J. Gen. Appl. Microbiol.* 24:51–58
181. Tecoma, E. S., Wu, D. 1980. *J. Bacteriol.* 142:931–38
182. Traub, W. H. 1982. *Chemotherapy* 28:363–68
183. Traub, W. H., Kleber, I. 1975. *Antimicrob. Agents Chemother.* 7:874–76
184. Uratani, Y., Kageljarna, M. 1977. *J. Biochem.* 81:333–41
185. Vaara, M. 1981. *J. Bacteriol.* 148:426–34
186. Vaara, M. 1983. *FEMS. Microbiol. Lett.* 18:117–21
187. Vaara, M., Vaara, T. 1981. *Antimicrob. Agents Chemother.* 19:578–83
188. Vaara, M., Vaara, T. 1983. *Antimicrob. Agents Chemother.* 24:107–13
189. Vaara, M., Vaara, T. 1983. *Antimicrob. Agents Chemother.* 24:114–22
190. Vaara, M., Vaara, T. 1983. *Nature* 303:526–28
191. Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurminen, M., et al. 1981. *FEBS Lett.* 129:145–49
192. Vaara, M., Vaara, T., Sarvas, M. 1979. *J. Bacteriol.* 139:664–67
193. Van Alphen, W., Lugtenberg, B., Berendsen, W. 1976. *Mol. Gen. Genet.* 147:263–69
194. Van Alphen, L., Lugtenberg, B., van Boxtel, R., Verhoef, K. 1977. *Biochim. Biophys. Acta* 466:257–68

195. Van Alphen, W., van Selm, N., Lugtenberg, B. 1978. *Mol. Gen. Genet.* 159: 75–83
196. Van Die, I., van Oosterhout, A., Bergmans, H., Hoekstra, W. 1983. *FEMS Microbiol. Lett.* 18:127–30
197. Verkeleij, A., van Alphen, L., Bijvelt, J., Lugtenberg, B. 1977. *Biochim. Biophys. Acta* 466:269–82
198. Voll, M. J., Leive, L. 1970. *J. Biol. Chem.* 245:1108–14
199. Voss, J. G., 1967. *J. Gen. Microbiol.* 48:391–400
200. Walker, C. A., Durham, N. N. 1975. *Can. J. Microbiol.* 21:69–74
201. Weigand, R. A., Rothfield, L. I. 1976. *J. Bacteriol.* 125:340–45
202. Weisser, R., Asscher, A. W., Wimpenny, J. 1968. *Nature* 219:1365–66
203. Weiss, J., Victor, M., Elsbach, P. 1983. *J. Clin. Invest.* 71:540–49
204. Weston, A., Brown, M. G. M., Perkins, H. R., Saunders, J. R., Humphreys, G. O. 1981. *J. Bacteriol.* 145:780–87
205. Whitfield, C., Hancock, R. E. W., Costerton, J. W. 1983. *J. Bacteriol.* 156: 873–79
206. Wilson, L. A. 1970. *Brit. J. Opthamol.* 54:195–213
207. Wilson, L. A., Spitznagel, J. K. 1968. *J. Bacteriol.* 96:1339–48
208. Wilson, M. E., Morrison, D. C. 1982. *Eur. J. Biochem.* 128:137–41
209. Wolf, M. K., Konisky, J. 1981. *J. Bacteriol.* 145:341–47
210. Wolf-Watz, H., Elmros, T., Normark, S., Bloom, G. D. 1975. *Infect. Immun.* 11:1332–41
211. Wright, S. D., Levine R. P. 1981. *J. Immunol.* 127:1146–51
212. Yamada, H., Mizushima, S. 1980. *Eur. J. Biochem.* 103:209– 18
213. Yem, D. W., Wu, H. C. 1978. *J. Bacteriol.* 133:1419–26
214. Yoshimura, F., Nikaido, H. 1982. *J. Bacteriol.* 152:636–42
215. Saske, S. K., Dockins, W. S., McFeters, G. A. 1980. *Appl. Environ. Microbiol.* 40:386–90
216. Zimelis, V. M., Jackson, G. G. 1973. *J. Infect. Dis.* 127:663–69
217. Zimmerman, W., Rosselet, A. 1977. *Antimicrob. Agents. Chemother.* 12: 368–76

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THE ACIDOPHILIC THIOBACILLI AND OTHER ACIDOPHILIC BACTERIA THAT SHARE THEIR HABITAT

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INTRODUCTION AND SCOPE

Many species of bacteria, in several genera, can acquire their energy for growth through the oxidation of inorganic sulfur. These bacteria, especially *Thiobacillus*, are corrosive agents due to the sulfuric acid that they generate. They do harm when, unrestrained, they attack pyrite in strip-mine coal (26, 45), and corrode concrete sewer conduits (64, 70), but they do useful service when domesticated to leach copper (3, 60), gold (14, 66), and uranium (7) from their ores. Additionally, they have potential as agents for the desulfurization of coal (12, 19, 34, 76) and the decontamination of industrial wastes (20, 63).

The acidophilic species of *Thiobacillus* and other acidophilic bacteria that share their habitat are the subject of this review. Acidophilism will be defined as the ability to grow optimally when inoculated into media poised at pH ≤ 3. This excludes *Thiobacillus intermedius*, which may lower the pH to below 3 (49) but will not grow when inoculated into media at this pH (29). Obligate thermophiles, the Archeabacteria such as *Sulfolobus* and *Thermoplasma*, are beyond the scope of this review, and no mention will be made of phototrophic microorganisms. Special attention will be given to bacteria that acquire their energy through the oxidation of ferrous iron. The relevant bacteria are gram-negative, aerobic rods that grow well at 20–30°C; one group will grow also at 50°C. All species grow well between pH 2 and pH 5 and poorly above pH 5.5; several species grow below pH 2. Their ecology and taxonomy will be discussed; new, previously unpublished research will be included. Little attention will be paid to details of metabolism, a subject covered by others (e.g. 17, 40, 65, 69, 83, and as collected essays in 61).

Transplants of these bacteria may be acquired from: (*a*) the American Type Culture Collection (ATCC), Rockville, MD. 20852, USA; (*b*) the German Collection (DSM), D-3400 Göttingen, F.R.G.; (*c*) the National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen AB9 8DG, Scotland; and (*d*) the Institute for Fermentation (IFO), Osaka 532, Japan.

CULTIVATION METHODS

Liquid Media

Vishniac & Santer (88) have listed numerous formulations with citations of origin. A medium modified from these sources is described below. It serves to cultivate several kinds of bacteria from acidic mineral environments, only the source of energy being changed for each kind. Basal mineral salts solution (MS) contains the following (% w/v): 0.2 $(NH_4)_2SO_4$, 0.01 KCl, 0.025 K_2HPO_4, 0.025 $MgSO_4 \cdot 7H_2O$, and 0.001 $Ca(NO_3)_2$. (The several times I omitted the calcium nitrate, growth was unaffected, but perhaps sufficient calcium was added with the inoculum.) The pH is adjusted with 1 N H_2SO_4, usually in the range of pH 2–4. Acidic MS will withstand autoclaving at 121°C.

Thiobacillus thiooxidans, also *T. ferrooxidans*, may be cultivated in MS containing 1% powdered sulfur. Sulfur melts at ~113°C, so the MS-sulfur slurry is sterilized by heating at 105°C for 1/2 hr on two successive days. Because the medium is at low pH, this manner of sterilization is adequate. When cultures are to be stored, or when large cell populations are desired, sterile powdered $CaCO_3$ may be added to hold the pH ~ 5.2. The $CaCO_3$ must not be sterilized in the aqueous medium. Rather, it is heated in the dry oven overnight at 160°C in aluminum foil packets.

Thiobacillus ferrooxidans and *Leptospirillum ferrooxidans* may be cultivated in MS containing $FeSO_4 \cdot 7H_2O$; 1% to 4% (w/v) being customarily used. In this review MS plus 4% $FeSO_4 \cdot 7H_20$ will be designated MS-Fe^{2+}. At pH greater than 1.6, the Fe^{2+} reacts with phosphate to form precipitate, and Fe^{3+} generated during growth forms insoluble ferric hydroxysulfate—not hydrated ferric oxide (limonite) as I, and others, have stated previously. The mineralogy of *T. ferrooxidans* cultures has been described by Lazarof et al (44). Stock cultures of iron-oxidizing bacteria are commonly maintained in Ms-Fe^{2+} at pH 3.5, the resulting precipitate being tolerated.

Thiobacillus acidophilus may be cultivated in MS-sulfur medium or in MS containing 0.1% glucose (or other organic compound) in lieu of sulfur. The glucose is prepared double-strength, is autoclaved, then is added aseptically to double-strength sterile MS at pH 3.5 after cooling.

Acidiphilium cryptum may be cultivated in MS enriched with 0.1% glucose and 0.01% yeast extract[1], pH 3.5. Other heterotrophic acidophiles may prefer different organic supplements.

Gelled Media

Thiobacillus thiooxidans has been grown as single colonies on acidified thiosulfate gelled with conventional agar; for example, see Reynolds et al (68). A more complex agar medium wherein fine colloidal sulfur is produced has been devised by Wieringa (93).

Thiobacillus ferrooxidans and *L. ferrooxidans* may be inhibited by conventional agar. Therefore various formulations require superior gelling agents. Manning (53) devised *ISP medium*, which uses Oxoid purified agar L28[2]. Phosphate is omitted so as to minimize mineral precipitate on the gel. I have used this medium successfully to isolate colonies of *T. ferrooxidans* (28), but not all strains form colonies on it. Growth may occur where cells are thickly deposited, yet not occur where they are thinly sown, as if re-enforcement were

[1]Dehydrated; purchased from BBL Microbiology Systems, Cockeysville, MD. 21030
[2]Purchased from Flow Labs, Rockville, MD. 20852

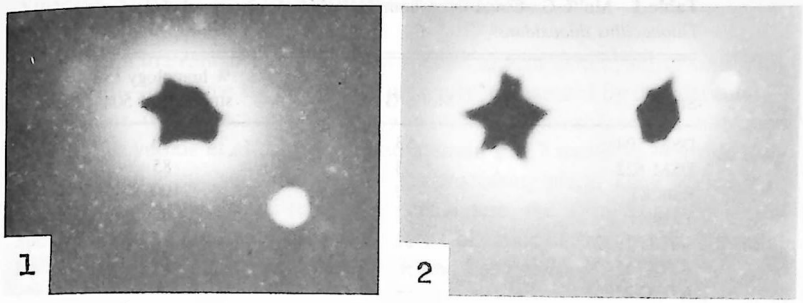
required by many cells in order that any one cell might grow. Thus single colonies may not appear. Mineral precipitate sometimes forms along the line of inoculation, to obscure growth or to be mistaken for it. Mishra et al (57) formulated a similar medium using Bapco agar (Japan) and agarose and carrageenan (Sigma) as gelling agents. They further reduced the concentration of the salts.

I have sought to more gently manipulate the inoculum and to avoid entirely surface mineral deposition by using a two-layer gel as in plating bacteriophage. MS, pH 2.5–4.5, containing 0.4% Sigma agarose, Type I, No. A-6013[3] is prepared by separately autoclaving double-strength acidic MS and double-strength agarose, mixing them after cooled to 45°C. Sufficient 40% $FeSO_4 \cdot 7H_2O$ (sterilized by filtration) is added to give a 1% solution of ferrous salt. After gentle mixing to avoid forming bubbles, ~20 ml of the molten medium is poured into Petri dishes, and this solidified *basal-layer medium* is stored at 10°C. The *over-layer* that receives the bacteria is prepared at the time of use, because agarose hydrolyzes when held at 45°C at low pH. Sixty mg agarose is added to 10 ml water in a 125-ml Erlenmeyer flask, is autoclaved at 121°C for 10 min, and is cooled to 45°. Ten-ml double-strength MS, also at 45°, prepared at proper pH and autoclaved in large (22 mm diameter X 180 mm) test tubes, is decanted into the molten agarose. Immediately, 0.5 ml of warm, sterile 40% $FeSO_4 \cdot 7H_2O$ are added. This medium is aseptically dispensed with a warm pipet into small (12 mm diameter X 100 mm) test tubes already at 42°C.

One tenth ml of iron-oxidizing bacteria appropriately diluted in sterile MS-Fe^{2+} is added with a pipet to the surface of molten over-layer in the small test tube. The bacteria are mixed into the molten medium by rapid rotation of the tube between one's palms, and the mixture is quickly decanted into a Petri dish containing the solidified basal-layer medium at 20°C. Immediately, the molten over-layer is spread over the solid basal-layer by quickly tilting the dish. The plates are inverted and incubated at 28°C. A pan of water in the incubator maintains high humidity therein.

Colonies appear after 1–4 weeks. They may be seen with the unaided eye, but they are more easily counted under a dissecting microscope at 10–20X. Colonies within the over-layer, viewed with light from beneath the microscope stage, are black, 0.5 mm diameter, and surrounded by a diffuse zone of "rusty" discoloration; colonies that grow on the surface are brilliant orange or yellow and several mm in diameter. The subsurface colonies appear white by reflected light, i.e. illuminated above the gel. Efficiency of plating is in the range of 50–80%, based on microscopic assay with a Petroff-Hausser bacteria-counting chamber. Figures 1 and 2 compare colonies of *T. ferrooxidans* and *A. cryptum* from mixed culture in liquid MS-Fe^{2+} medium. The role of *A. cryptum* with *T. ferroxidans* will be described.

[3]Purchased from Sigma Chem. Co., Box 14508, St. Louis, MO. 63178



Figures 1 & 2 *T. ferrooxidans* Lp and *A. cryptum* Lhet2 from mixed culture in MS-Fe^{2+}, plated on double-layer Fe^{2+} agarose. Magnification is 20X, with illumination from beneath the gel. *T. ferrooxidans* colonies are black, 0.5 mm diameter, surrounded by a diffuse rust-colored zone seen here as a bright area. *A. cryptum* colonies are buff-white, smooth, 0.1 to 0.3 mm in diameter.

ACIDOPHILES THAT UTILIZE SULFUR BUT NOT IRON AS SOURCE OF ENERGY

Thiobacillus thiooxidans

T. thiooxidans was discovered and named by Waksmann & Joffe (89, 90). Starkey (78, 79) extended its description. An abundant literature exists on this and other Thiobacilli (35, 42, 43, 77, 88). *T. thiooxidans* is the single species of Type III *Thiobacillus* tabulated by Vishniac in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (87). This dilineation of Thiobacilli into Types I, II, and III is based on the composition of their cellular lipids. Katayama-Fujimura et al (39) continue analyses of cellular lipids and extend their study to include the ubiquinones within this, and other, species.

Strains presently labeled *T. thiooxidans* have vastly differing morphology and behavior in MS-sulfur. Cells of some strains attach themselves to sulfur particles. If motile, they propel the sulfur particle at great speed, though the particle exceeds by many times the mass of the cell. Some other strains appear not to attach to sulfur particles, but they oxidize sulfur equally well. Schaeffer & Umbreit (71) identified phosphotidylinositol as a "wetting" agent of sulfur in a *T. thiooxidans* culture.

The mol% G+C of *T. thiooxidans* DNA is commonly cited as 50–52 (36, 87), but strain DSM 612 isolated by Hans Hippe (Göttingen) from acidic soil in the Netherlands contains 62 mol% G+C, Table 1, and shows negligible DNA homology with other strains examined. It may be similar to *T. albertis* (11a). Sulfur-oxidizing acidophiles that at present bear the binomial, *T. thiooxidans*, probably represent more than one species, as I have already remarked (28). *T. thiooxidans* also grows in acidic thiosulfate medium.

Table 1 Mol% G+C and DNA homologies among strains labeled *Thiobacillus thiooxidans*[a]

Strain	Mol% G+C	% homology to strain DSM 504
DSM 504	53	100
DSM 622	53	85
Ram 8T	53	100
T219	53	89
ATCC 19377[b]	52	87
ATCC 15494	—	87
ATCC 21835	—	79
DSM 612	62	11, 8

[a] DNA was extracted from cultures grown in MS-sulfur at pH 3. Methods are given elsewhere (28). From published (28) and unpublished research by the author.
[b] Type Strain of *Thiobacillus thiooxidans*.

Ambiguity in Establishing Thiosulfate Utilization at Low pH

Sulfur chemists repeatedly have shown that thiosulfate decomposes in mild acid (15, 25, 51, 72, 94). This is easy to confirm with a 1% solution of $Na_2S_2O_3 \cdot 5H_2O$ at pH 4. Sparkling clear, the solution may be filter-sterilized and in other ways manipulated at room temperature. But sulfur is forming; first nascent, then colloidal, it is seen as a faint turbidity after an hour when viewed against the dark surface of the laboratory bench. The equilbrium between thiosulfate and its decomposition products is complex. The products include elemental sulfur (S_6 and S_8), H_2S, SO_2, and various polythionic acids (15, 72). Decomposition is faster at lower pH and at higher temperature. In cultures, the colloidal sulfur may be mistaken for bacterial growth, especially because Thiobacilli lower the pH as they grow. Disappearance of thiosulfate concomitant with growth will occur if the bacteria consume any one of the several decomposition products, as equilbrium is continually re-established between these products and thiosulfate.

Conclusions derived from elaborate quantitative measurements of "thiosulfate utilization" in acid remain ambiguous when the qualitative premise to support these measurements is erroneous. I do not presume to claim that *T. thiooxidans* is incapable of utilizing thiosulfate, rather I ponder what these elaborate measurements prove. Perhaps it was his difficulty in evaluating alleged thiosulfate (and tetrathionate) consumption in cultures at changing pH that led Vishniac (87) to the extreme pronouncement: "—the ability to use certain sulfur compounds—is not a useful diagnostic feature." The preceding difficulty is avoided at pH 6–7 where thiosulfate is stable and where thiosulfate utilization has been established in the nonacidophilic Thiobacilli.

Species Similar to Thiobacillus thiooxidans

It is difficult to evaluate claims for new species of acidophilic, sulfur-oxidizing Thiobacilli—the Type III Thiobacilli presently represented by the binomial *T. thiooxidans* (87)—because variability within *T. thiooxidans* has not been defined. Reynolds et al (68) described a strain (51.7 mol% G+C) that they isolated from acidic soil adjacent to a gas-processing plant. They named it *T. kabobis*. According to the rules of nomenclature, the Type Strain of a new species must be deposited in a Type Culture Collection in order that its binomial be valid. *T. kabobis* was not deposited, and has been lost.

ACIDOPHILES THAT UTILIZE IRON AS SOURCE OF ENERGY

Thiobacillus ferrooxidans

Thiobacillus ferrooxidans was discovered and named by Colmer and coworkers (13, 82). Later Leathen et al (46) isolated an iron-oxidizing bacterium that they claimed was unable to utilize sulfur, and they named it *Ferrobacillus ferrooxidans*. But careful work by Kelly & Tuovinen (41) demonstrated that this isolate did utilize sulfur, thus invalidating the new binomial.

A systematic study of a large collection of mesophilic iron-oxidizing bacteria was made in my laboratory (28). These bacteria were shown to comprise seven different DNA homology groups. The analyses have been extended to include *T. ferrooxidans* strain TM (= ATCC 13661) and the Type Strain (ATCC 23270); the results are summarized in Table 2. One strain, m-1 (Homology Group No. 7), does not utilize elemental sulfur as sole energy source. Strain BU-1 (Homology Group No. 1), representative of isolates from copper leaching sites in Bulgaria (22), has been discovered to be similar, or identical, to *Leptospirillum ferrooxidans* (Hans Hippe, Göttingen; personal communication). It also does not grow in MS-sulfur. Strain BU-1 is similar to a transplant of *L. ferrooxidans*, kindly provided by G. A. Zavarzin (Moscow), that I designate herein as strain Z-2.

Leptospirillum ferrooxidans

Leptospirillum ferrooxidans was named and described by Markosyan (54, 55) and further characterized by Balashova et al (2). This acido-mesophile grows more slowly in MS-Fe^{2+} than *T. ferrooxidans*, does not grow in serial culture in MS-sulfur, and is actively motile. By comparison, *T. ferrooxidans* is sluggishly motile, if motile at all. *Leptospirillum* occurs as curved rods, whereas *Thiobacillus* occurs as straight rods.

Table 2 Mol% G+C and DNA homologies among strains of acidophilic iron-oxidizing bacteria[a]

Strain	Mol% G+C	% Homology to: BU-3	WVa	DECp	19859	PH	m-1	DNA homology group
BU-3, BU-1, (= DSM 2391), BU-2, BU-5	55–56[b]	87–100	0	6–11	12–16	0	0–9	No. 1
WVa, Lp (= IFO 14245), D-26, C-52, A-6, BA-4	56–57	0	78–100	18–38	24–41	14–32	0	No. 2
DECp, A-4, 11Fe, TIO	57–58	0	17–38	56–100	58–65	13–23	0–9	No. 3b
ATCC 19859, ATCC 23270[c], ATCC 13598p, ATCC 13728, ATCC 13661, DSM 583, F-221	58–59	0	18–46	38–52	85–100	0–38	0–9	No. 3a
PH (= IFO 14262)	59	0	34	12	17	100	0	No. 4
m-1 (= DSM 2392)	65	8	0	10	0	6	100	No. 7

[a] From published (28) and unpublished research by the author. DNA homology groups No. 5 (strain SOC) and No. 6 (strain AGN) are not included. See Ref. 28 for methods and for data on individual strains.

[b] Determined by reverse-phase, high-pressure, liquid chromotography after enzymatic hydrolysis by C. W. Gehrke and coworkers (per. comm.) See Ref. 28. Other determinations were by the author with the DNA melting point method.

[c] Type Strain of *Thiobacillus ferrooxidans*.

Facultative Thermophilic Iron-Oxidizing Bacteria

Facultative thermophilic iron-oxidizing bacteria, first encountered from an Icelandic hot spring, were discussed in 1977 by Le Roux et al (47). Other strains from copper leaching dumps were described by Brierley & Lockwood (8). These acidophiles have been further studied by Norris et al (62). They grow in the range of 10–35°C (like *T. ferrooxidans*), but also grow as high as 55°, if the Fe^{2+} medium is supplemented with "either yeast extract, organic compounds with sulfhydryl groups (e.g. glutathione, cysteine), or an organic compound (e.g. glucose or serine) plus a source of sulfur (such) as flowers-of-sulfur, thiosulfate, tetrathionate, pyrite or nickel sulfide" (10). In this circumstance they do not fix CO_2 or reductively assimilate sulfate. In other circumstances they can fix CO_2 and use some sulfur source other than sulfate for anabolic metabolism while using Fe^{2+} as source of energy, but they cannot use elemental sulfur as sole source of energy (J. A. Brierley, personal communication).

Table 3 categorizes both the acido-mesophiles and the acido-facultative thermophiles, providing an operative classification. Observe that the mol% G+C of the four groups is distinctive for each group, emphasizing their phenotypic distinctiveness. *Leptospirillum ferrooxidans* and strain m-1 grow much more slowly than *T. ferrooxidans* in MS-Fe^{2+}. But if this medium is supplemented with 0.01% (w/v) cysteine, they grow as fast as typical *T. ferrooxidans*.

However, neither *L. ferrooxidans* nor m-1 grow at 50°C with, or without, cysteine. As iron-oxidizers that cannot grow through serial culture in MS-sulfur, they differ from all *T. ferrooxidans*. (One strain of *T. ferrooxidans*, D-26, previously reported as a sulfur nonoxidizer, 28, has since been successfully adapted to grow in MS-sulfur.) Their apparent inability to utilize powdered sulfur as sole source of energy needs to be scrutinized. Will they utilize inorganic sulfur if provided an appropriate organic supplement? Experiments by the author with 0.02% cysteine as supplement in MS-sulfur were inconclusive; this work is to be continued. Also noteworthy is the fact that these two organisms do not form colonies on the double-layer agarose medium, with or without cysteine, whereas all strains of *T. ferrooxidans* examined (twenty strains) do form colonies on this medium.

Interest in the iron-oxidizing bacteria derives from their importance in bio-corrosion generally, and from their use in leaching metals from ores specifically. Most research into mineral leaching has been with cultures labeled *T. ferrooxidans*, sometimes with imperfectly defined mixtures of strains. *T. ferrooxidans* is believed to participate in the leaching of pyrite (FeS_2) by direct oxidation of this mineral. In the commercial leaching of copper and uranium from their ores, *T. ferrooxidans* may act indirectly. The Fe^{3+} formed by *T. ferrooxidans* from FeS_2 associated with these ores acts as nonbiotic oxidant of copper and uranium. Recently, however, DiSpirito & Tuovinen (16) described direct oxidation of uranous ion coupled to CO_2-fixation by *T. ferrooxidans*. Growth of *T. ferrooxidans* solely with uranous ion in MS has yet to be recorded. *L. ferrooxidans* oxidizes FeS_2 only in mixed culture with sulfur-oxidizing bacteria (2). Strain m-1, also a sulfur nonoxidizer, has not been examined in slurries of FeS_2.

Additional information, dealing with metabolism and geochemistry, has been compiled (61). Current research will appear in *Planetary Ecology* (10a).

ACIDOPHILES IN MIXED AND IN PURE CULTURES

Circa 1963, Lundgren and coworkers (50) claimed that *T. ferrooxidans* could be adapted to grow with glucose substituting as energy source for Fe^{2+}. Shafia et al (73), 1969–72, trained *T. ferrooxidans* strain BCU-4 to grow on glucose that, as a result, lost its ability to utilize Fe^{2+}. They referred to the glucose-adapted acidophile as a "heterotrophic convertant," and they labeled it KG-4. They did not report whether it could utilize sulfur as sole source of energy.

Zavarzin (95) in 1972 called attention to close association between acidophilic heterotrophs and autotrophic *T. ferrooxidans* in natural habitat. He suspected that KG-4 was a contaminant, not a "convertant," mutant, or physio-

Table 3 Acidophilic iron-oxidizing bacteria[a]

	Taxonomic group and representative strain(s)			
	I	II	III	IV
	BU-1 (= DSM 2391)	ATCC 23270 (Type Strain)	m-1 (= DSM 2392)	TH1, TH3
Genus	*Leptospirillum* (Markosyan)	*Thiobacillus* (Beijernick)	Not named	Not named
Species	*L. ferrooxidans*[b] (Markosyan)	*T. ferrooxidans* (Temple & Colmer) and unnamed species	Not named	Not named
Description				
Mol% G+C[c]	51–56	57–62	65	48
DNA Homology Group[c]	No. 1	ATCC 23270 = No. 3a; Subspecies = No. 3b; Other species = Nos. 2, 4, 5 and 6.[d]	No. 7	
Use powdered sulfur as source of energy through serial cultures[e]	No	Yes	No	No
Morphology	Curved rods	Straight rods	Straight rods	Straight rods
Motility, pH 3.5[f]	+++	+ or −	−	
Generation time (hr) in batch culture, pH 1.8	≥12	8±2	≥12	
Growth at 50°C	No	No	No	Yes[g]
Colonies on Fe^{2+} agarose[h]	Negligible	Subsurface: 0.1 to 0.5 mm diameter, black, surrounded by "rusty" diffuse discoloration. Surface: several mm diameter, orange colored.	Negligible	

[a] All strains are gram-negative aerobic rods that can use Fe^{2+} as sole source of energy at 25°C.

[b] Strain Z-2 (my designation), which was received from G. A. Zavarzin labeled *Leptospirillum ferrooxidans*, contained 51.7 mol% G+C as determined by reverse-phase, high-pressure, liquid chromotography after enzyme hydrolysis of its DNA by C. W. Gehrke and coworkers (personal communication). Strain BU-1 is similar, or identical, in phenotype to strain Z-2, and contains 55.6 mol% G+C as determined by the same method.

[c] See Table 2 and Ref. 28. The mol% G+C for Taxonomic Group IV is from Brierley et al (9).

[d] For the present, strains in Homology Group Nos. 3a, 3b, 2, 4, 5, and 6 can all be considered *T. ferrooxidans*, but they should be recognized as separate candidate species, and the Type Strain, ATCC 23270, affixes the binomial, *T. ferrooxidans*, to DNA Homology Group No. 3a.

[e] Possible simultaneous oxidation of Fe^{2+} and powdered sulfur has not been adequately investigated.

[f] +++ = highly motile, + = poorly motile and variable, − = not motile.

[g] An inorganic compound of reduced sulfur is required for autotrophic growth when Fe^{2+} is used as the source of energy.

[h] In the double-layer Sigma agarose medium described herein.

logical variant. Guay & Silver (23) in 1975 isolated a contaminant from *T. ferrooxidans* strain TM, which they named *Thiobacillus acidophilus*. This acidophile can utilize either powdered sulfur or glucose (and other organic compounds) as source of energy but cannot grow using Fe^{2+} as sole energy source. *T. acidophilus*, maintained in my laboratory five years and tested periodically, has never shown capability of utilizing Fe^{2+} for energy, confirming the original description of this species (23). (In phenotype, *T. acidophilus* more closely resembles *T. thiooxidans* than *T. ferrooxidans*.) Mackintosh (52) also concluded that *T. ferrooxidans* cultures were apt to be contaminated.

One wonders, in retrospect, why the warnings inherent in the findings by G. A. Zavarzin, and the others, did not elicit suitable care in microbiological technique to prevent mixed cultures from being mistaken for pure ones. But attempts to detect heterotrophic contaminants sometimes failed when conventional organic media were acidified for that purpose. That heterotrophs from lean mineral environments might be "adapted" to grow only in low concentration of organic nutrient was generally not recognized. Had organic media been diluted as well as acidified, the common hidden acidophile, *Acidiphilium cryptum*, may have been detected. *A. cryptum*, in fact, grows on agar gels without added organic nutrient, presumably consuming organic impurities in, or absorbed to, the agar medium. The "heterotrophic convertent," KG-4, is an early isolate of *Acidiphilium*, as I already have demonstrated (27, 32). I shall not debate, one by one, past claims for "mixotrophy" by *T. ferrooxidans*. Rather, Table 4 lists pairs of autotroph and heterotroph of established pedigree that previously were presumed to be pure *T. ferrooxidans*.

What is the Evidence for Heterotrophic Growth by Thiobacillus ferrooxidans?

Unlike common heterotrophic bacteria that have many conveniently measureable phenotypic traits to mark their identity, *T. ferrooxidans* has few such traits. Therefore, DNA analyses have been used to establish purity of a culture. The glucose- and Fe^{2+}-grown cultures must yield cells with identical mol% G+C and high DNA homology in order to authenticate these two cultures as the same species. Observe the contrary result in Table 4. Thiobacilli that grow either with inorganic sulfur or organic compounds as source of energy do exist among nonacidophilic representatives of the genus: *T. intermedius* (69), *T. novellus* (65a, 69), and *T. versutus* (*Thiobacillus* A2) (43, 81). We might anticipate an acidophilic counterpart.

Recently, D. R. Woods (personal communication, 2a) reported a mixotrophic *T. ferrooxidans*, strain FD1, that can grow as lithotroph-utilizing Fe^{2+} or as a mixotroph simultaneously utilizing both Fe^{2+} and glucose but cannot grow as organotroph solely with glucose. (As a mixotroph, perhaps it grows as

Table 4 Examples of mixtures of autotroph and heterotroph previously presumed pure *Thiobacillus ferrooxidans*[a]

Example number	Citation for impurity	Pedigree of mixture	Mol% G+C[b]	Intrapair DNA homology[b]
1	Guay & Silver (23)	*T. ferrooxidans* TM (= ATCC 13661) *T. acidophilus* ATCC 27807 (= DSM 700)	56 63–64	N.D.
2	Harrison (27, 29, 32)	*T. ferrooxidans* BCU-4 *Acidiphilium* KG-4p (= DSM 2390)	N.D. 68	N.D.
3	Harrison (27, 29, 32)	*T. ferrooxidans* Lp (= IFO 14245) *A. cryptum* Lhet2 (= ATCC 33463 = DSM 2389, = NCIB 11690)	57 69–70	0
4	Harrison (27, 29, 32) Arkesteyn & de Bont (1)[c]	*T. ferrooxidans* ATCC 13598 *A. cryptum* 13598het (= NCIB 11691)	59 70	0
5	Harrison, unpublished	*T. ferrooxidans* AP-44 *Acidiphilium* sp. 44het (= DSM 2613)	57 70	N.D.
6	Johnson & Kelso (38)	*T. ferrooxidans* NCIB 8455 *Acidiphilium* sp. (= NCIB 11822)	59 N.D.	N.D.
7	Johnson & Kelso (38)	*T. ferrooxidans* NCIB 10435 *Acidiphilium* sp. (= NCIB 11745 and 11746)	N.D. N.D.	N.D.

[a] These are only those examples studied in detail. Moreover, allegedly pure *T. ferrooxidans* sometimes is contaminated with mold, yeast, *T. thiooxidans*, or another strain of *T. ferrooxidans*. See Ref. 28.
[b] All DNA analyses were by the author except those in Example No. 1, which are from Guay & Silver (23). N.D. = not determined.
[c] These investigators mislabeled the heterotroph *T. acidophilus*. *A. cryptum* had not been described at the time of their study. See Ref. 29.

a lithotrophic heterotroph, using Fe^{2+} for energy and glucose for carbon.) These workers claim that utilization of glucose by strain FD1 is tightly dependent upon Fe^{2+} concentration: at high concentration glucose is not utilized; as Fe^{2+} diminishes in concentration, Fe^{2+} and glucose are utilized simultaneously. They suggest that this restrictive mixotrophy may, in the past, have been overlooked because the T. *ferrooxidans* strains may have been cultivated at a concentration of Fe^{2+} too high to permit utilization of glucose. Indeed, glucose is somewhat inhibitory to growth of many strains of *T. ferrooxidans* at the Fe^{2+} concentration commonly used: 4% $FeSO_4 \cdot 7H_2O$ (w/v). Gotteschal & Kuenen (21) suggested the use of a chemostat, containing organic and inorganic substrate, to select for facultatively chemolithotrophic Thiobacilli.

All my attempts to entice several strains of *T. ferrooxidans* to grow solely with glucose have failed. The autotroph disappears in these series of glucose cultures as predicted by dilution, allowing for some growth in the first one or two serial passages on the residual Fe^{2+} added with the inoculum. Borichewski & Umbreit (6), however, have claimed to grow *T. thiooxidans* with glucose replacing sulfur, where provision was made to remove intermediates of glucose catabolism allegedly inhibitory to the acidophile. The hypothetical inhibitor was removed via membrane tubing in batch culture, much like dialysis is used to cleanse the blood of patients with kidney malfunction. I sought a similar result using *T. ferrooxidans,* but I did not succeed.

Furthermore, I cultivated *T. ferrooxidans* in mixed culture with *T. acidophilus,* separated by a membrane barrier, wherein *T. ferrooxidans* failed to grow with glucose in lieu of Fe^{2+}.(The assumption had been that *T. acidophilus* would consume the hypothetical inhibitory metabolite of glucose catabolism, thus allowing growth of *T. ferrooxidans* to be sustained.) I am unaware of any confirmation of the claim of Borichewski & Umbreit for heterotrophic growth by *T. thiooxidans*. To date, no strain of *T. ferrooxidans* has been proven capable of growth solely on glucose in lieu of Fe^{2+}. Such a strain awaits discovery.

THE ROLE OF PLASMIDS Plasmids have been observed in *T. ferrooxidans* (56). In other bacterial groups plasmids have been shown to slightly alter cell phenotype (11), but their role in acidophilic Thiobacilli has not been established and has nothing to do with the contamination of *T. ferrooxidans* illustrated in Table 4. Algebraic equations demonstrate that a hypothetical plasmid cannot account, structurally, for a bacterium with 57 mol% G+C to change to one containing 70 mol% G+C. To do so, a plasmid with 80 mol% G+C must comprise 56% of the total bacterial DNA! For this same reason, D. P. Kelly (84) early discounted plasmids as explanation for the observed differences in mol% G+C of glucose- and Fe^{2+}-grown cells.

How do Heterotrophs Maintain Themselves in Mineral Media with Thiobacillus ferrooxidans?

Distilled water held for long periods in glass or plastic vessels may sustain growth of mold and, if held in the light, algae. Water "demineralized" with ion-exchange resins is especially rich in organic matter. Shehata & Marr (74) stressed the difficulty in freeing water from organic substances. Bacteria grow in mineral salts where no organic material is overtly added (5, 67). In mineral salts solution *Escherichia coli* may attain a population of 10^6 cells/ml with regularity, presumably the result of consuming organic impurities in the salts as well as those absorbed from the utensils and the atmosphere. The MS-Fe^{2+} medium described herein to cultivate *T. ferrooxidans* contains over 4% salts (w/v). *A. cryptum,* an obligate chemoorganotroph, may be cultivated in this medium through serial cultures at a population of 0.5 to 1 $\times$ 10^7 cells ml^{-1} (32).

It has not been possible to identify a particular inorganic component as the principle source of organic impurity. Rather, with the removal of each particular salt, one by one, the population of *A. cryptum* diminishes proportionally, though the ferrous sulfate is particularly stimulatory. Tiny colonies form on this medium gelled with agarose, Figure 3. There is no evidence for utilization of $(NH_4)^+$, nor for the utilization of Fe^{2+} as source of energy. At a population of 0.5 to 1 $\times$ 10^7 cells ml^{-1}, *T. ferrooxidans* and *L. ferrooxidans* consume appreciable Fe^{2+}, measured by titration with 0.01 M $Ce(NH_4)_4(SO_4) \cdot 2H_2O$ using 1,10-Phenanthroline as redox indicator (31).

Conversely, *A. cryptum* at ten-fold this population gives no evidence for consumption of Fe^{2+} under identical conditions (unpublished experiments by the author). (The *A. cryptum* cells were harvested at the centrifuge, then suspended in MS-Fe^{2+} medium.) Bubbling nitrogen or hydrogen together with air through these MS-Fe^{2+} cultures increases neither the growth rate nor the population of *A. cryptum* (strain Lhet2) over that obtained through bubbling

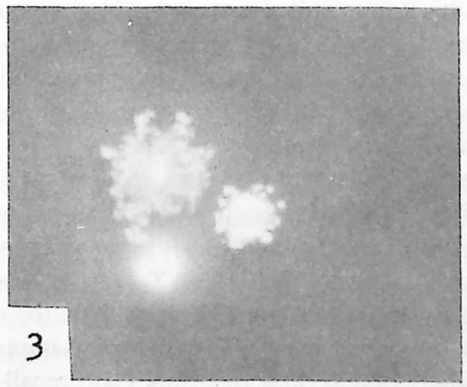


Figure 3 *A. cryptum* Lhet2 from pure culture in MS-Fe^{2+} plated and photographed as described in Figures 1 & 2. Observe the two lobate colonies.

with air alone. Presumably, like *E. coli*, the heterotrophic acidophile grows by consuming organic impurities in the inorganic medium. D. B. Johnson (38) also has observed persistent growth in mineral salts by heterotrophs isolated as contaminants from *T. ferrooxidans* cultures and isolated in "acid streamers" from a pyrite mine in Wales. He also concludes that these acidophiles grow on organic impurities in the salts and absorbed from the atmosphere. Utilization of atmospheric organic matter by chemoorganotrophs in mineral media also has been discussed by A. J. Kluyver in relation to early (1903) observations by Beijerinck and van Delden (41a).

Enterobacter aerogenes forms colonies on MS, pH 6, gelled with agarose (Sigma No. A-6013). If a liquid culture is plated out on this medium, the number of colonies per ml is the same, within statistical variance, as the number of cells per ml in the liquid culture as determined with the microscope using a Petroff-Hausser counting chamber (unpublished experiments by the author). The colonies are barely visible to the unaided eye, but are easy to count under the dissecting microscope at 10X. Thus, these gells do contain nutrient for heterotrophic bacteria. More will be said on this matter in a subsequent section, where the organic material in agar is shown to be inhibitory to *T. ferrooxidans*, and where this inhibition is removed by *A. cryptum*.

In the preceding experiments the heterotroph was examined in pure culture. We have not considered organic material added to the medium by a "prime producer" such as *T. ferrooxidans*. In fact, all bacteria add organic material to the medium in which they are suspended. Fragments of pili, fragments of flagella, and other surface material will accumulate. In addition, metabolites may elute from the cell. No membrane is a perfect "valve." *Enterobacter aerogenes* suspended in 0.03 M sodium phosphate, pH 7.1, at 40°C (growth temperature) loses cell material in absence of cell lysis, and this material can be recovered by *E. aerogenes* cells to be used by them as nutrient (33). It is well known that the lag phase measured on the bacterial growth curve is shortened in proportion to the population of the inoculum. Lodge & Hinshelwood (48) have demonstrated that this is the result of stimulation by compounds eluted from the cells. Soil enrichment cultures of *Azotobacter* in medium lacking added nitrogen are difficult to free from bacterial, and even protozoal, contaminants that thrive on the nitrogen compounds acquired from the nitrogen-fixing bacteria. *T. ferrooxidans* may, itself, fix nitrogen (52).

Moreover, Schaeffer & Umbreit (71) have demonstrated that *T. thiooxidans* loses 10–15% of fixed CO_2 in absence of cell autolysis or breakdown. There is no reason why *T. ferrooxidans* cannot also lose organic material to the growth medium. [Fatty acids (10^{-2} to 10^{-1} M) in *T. ferrooxidans* cultures provoke leakage of phosphate, sugar, and nucleic acids from the cells (86)]. Culture filtrates from *T. ferrooxidans* can provide enrichments necessary for the growth of the facultative-thermophilic iron oxidizers (10); recall that the latter acidophiles cannot grow at 55°C in Fe^{2+} medium without organic supplement. We

should not be surprised that *A. cryptum* maintains itself in MS-Fe^{2+}, especially as cohabitat with *T. ferrooxidans*.

Figures 1 and 2 each display a colony of *A. cryptum* near colonies of *T. ferrooxidans*. These platings were made from mixed cultures of the two species. When near colonies of the autotroph, *A. cryptum* colonies tend to be smooth; but when well-isolated therefrom, or when from pure culture, they tend to be lobate (see Figure 3). These cultures of *A. cryptum*, both the pure one and the one mixed with *T. ferrooxidans*, had been maintained through numerous serial transfers in MS-Fe^{2+} where no organic material had been overtly added.

Purifying Mixed Cultures

Acidiphilium cryptum that contaminates *T. ferrooxidans* cultures may easily be isolated. The mixture is streaked on an agar gel containing MS enriched with 0.1% glucose and 0.01% yeast extract, as formulated in a previous section of this review. Colonies are smooth, white, brownish, or pink, and 1 mm to several mm in diameter after 1–2 weeks' incubation at 28°C. Because the gel is contaminated with dormant *T. ferrooxidans*, it is prudent to repeat this procedure one or more times. Colonies may appear also on MS-Fe^{2+} agarose gels, but they are much smaller. See Figures 1, 2, and 3.

Acquiring autotrophic *T. ferrooxidans* in pure culture is more difficult. Five methods are available:

1. *Plating*. For those strains that produce colonies on one of the plating media herein described, a colony is selected and cultivated in liquid MS-Fe^{2+}. It is again plated, and a single colony is again selected to ensure purity. Some strains, however, form colonies poorly. I have never acquired colonies from cultures of *Leptospirillum ferrooxidans*.
2. *Selection by acid*. Most contaminating heterotrophs do not grow below pH 2, whereas most autotroph *T. ferrooxidans* and *L. ferrooxidans* grow at pH $\leq$ 1.7. Rapid, serial cultivation of the mixture in MS-Fe^{2+} at pH $\leq$ 1.9 enriches for the iron-oxidizer, and the heterotroph eventually is diluted out, although this may require six or more serial transfers through growth medium. Ideally, this method should be combined with the following method.
3. *Statistical dilution*. Microscopic assay is made to determine the number of cells per ml. Then the culture is diluted into multiple tubes containing sterile MS-Fe^{2+} so that most tubes receive no cells and it is improbable that any tube receives more than one. After growth appears in one of the tubes, this culture is assayed as before, and the procedure is then repeated one or more times. This method has the advantage that in mixtures of two or more strains of *T. ferrooxidans*—these mixtures are seldom detectable by plating or microscopic examination—the predominant strain of *T. ferrooxidans* is

selected from among the others. Fresh enrichment cultures may contain more than one strain of *T. ferrooxidans*. Guay et al (24) observed changes in mol% G+C concomitant with change in the sulfide substrate, and I have observed a similar result when cultures were transferred from MS-Fe^{2+} to MS-sulfur medium (28). Such changes in mol% G+C will occur when a culture comprises two or more strains of *T. ferrooxidans*, or when *T. ferrooxidans* is mixed with another species.

4. *Single-cell isolation*. A micro-manipulator may, of course, be employed to isolate single cells, but this is a tedious procedure and is subject to contamination.
5. *Selection with heavy metals*. D. B. Johnson & W. I. Kelso (38) used 1–5 g Cu^{2+} per liter in MS-Fe^{2+} in serial cultures to enrich for *T. ferrooxidans*, based upon the observation by Tuovinen et al (85) that the autotroph readily adapts to tolerate heavy metal ions. Apparently, the heterotroph does not so readily adapt.

Enhancement of Iron Oxidization Caused by Heterotrophic Acidophiles

It is well established that pyruvate inhibits acidophilic Thiobacilli; see, for example, the review by Matin (58). Pyruvate inhibition may be overcome by the presence of acidophilic heterotrophs in mixed culture with autotrophic *T. ferrooxidans* (30). Table 5 demonstrates that enhancement of iron-oxidization by autotrophic *T. ferrooxidans* in mixed culture with *A. cryptum* is due to the consumption of the inhibitor (pyruvic acid or derivative) by *A. cryptum*. The same result is obtained with *T. acidophilus* substituting for *A. cryptum*.

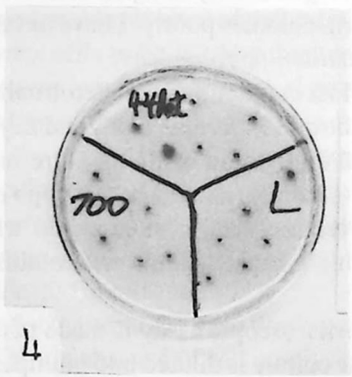


Figure 4 The 9 cm–diameter Petri dish contains MS plus 0.5% $FeSO_4 \cdot 7H_2O$, pH 3, gelled with 1.5% agar (No. 11849; BBL Microbiology Systems, Cockeysville, MD. 21030). Approximately 10^8 cells of *T. ferrooxidans* AP-19 were spread over the surface of the gel. After this inoculum was absorbed by the gel, heterotrophic bacteria were deposited with a pin at points on the gel. Legend: *L* = *A. cryptum* Lhet2; *44het* = a strain of *Acidiphilium* from allegedly mixotrophic *T. ferrooxidans* (Table 4); *700* = *T. acidophilus* DSM 700. The dark points of growth contain the pair of species, both autotroph and heterotroph. The small white spots are bubbles in the gel.

Table 5 *Inhibition of T. ferrooxidans by pyruvic acid is overcome in mixed culture with A. cryptum*[a]

Test condition[b]		Additions[c]	Result after incubation for:[d] zero days[e]	3 days	4 days	6 days
A.	Sterile medium was incubated 6 days and then filtered for use.	1. T	−−; 1×10^6	++	+++; 2.5×10^8	+++; 2×10^8
		2. T P	−−; 1×10^6	−−	−−; $<5\times10^6$	−−; $<5\times10^6$
		3. T P A	−−; 5×10^6	±	+; 6×10^7	+++; 2×10^8
		4. P A	−−; 4×10^6	−−	−−; $\sim5\times10^6$	−−; $\sim5\times10^6$
B.	Sterile medium was inoculated, incubated 6 days, and then filtered for use.	1. T	−−; 1×10^6	++	+++; 2.5×10^8	+++; 2×10^8
		2. T P	−−; 1×10^6	−−	−−; $<5\times10^6$	−−; $<5\times10^6$
		3. T P A	−−; 5×10^6	±	+; 2.5×10^7	+++; 1.5×10^8
		4. P A	−−; 4×10^6	−−	−−; $\sim5\times10^6$	−−; $\sim5\times10^6$
C.	Sterile medium was inoculated with *A. cryptum* and 0.004% sodium pyruvate, incubated 6 days, and then filtered for use.	1. T	−−; 1×10^6	++	+++; 2×10^8	+++; 2×10^8
		2. T P	−−; 1×10^6	−−	−−; $<5\times10^6$	−−; $<5\times10^6$
		3. T P A	−−; 5×10^6	±	+; 3.5×10^7	+++; 1.5×10^8
		4. P A	−−; 4×10^6	−−	−−; 1.5×10^7	−−; 1×10^7
D.	Sterile medium was inoculated with 0.004% sodium pyruvate, incubated 6 days, and then filtered for use.	1. T	−−; 1×10^6	−−	−−; $<5\times10^6$	−−; $<5\times10^6$
		2. T P	−−; 1×10^6	−−	−−; $<5\times10^6$	−−; $<5\times10^6$
		3. T P A	−−; 5×10^6	−−	+; 6×10^7	+++; 1.5×10^8
		4. P A	−−; 4×10^6	−−	−−; 1.5×10^7	−−; 2×10^7

[a] Unpublished research by the author.

[b] Ten ml sterile medium, MSFe^{2+}, pH 2.5, in test tubes (18 mm diameter × 150 mm), was incubated at 28°C at an angle of 20° above horizontal. Sterilization of the medium after 6 days' incubation was with a membrane filter. An uninoculated tube of each medium served as an additional control but is not tabulated. Each of the 5 test tubes of each medium were prepared in duplicate, 40 tubes being examined during the experiment.

[c] Additons were: T = *T. ferrooxidans* strain Lp; P = 0.004% sodium pyruvate; and A = *A. cryptum* strain Lhet2.

[d] −− = no "rusty" discoloration greater than in the uninoculated control; ± = discoloration barely discernible; +++ = maximum degree of discoloration. The numerals represent cells ml^{-1} (microscopic assay).

[e] Cells ml^{-1} at time of inoculation (0 days) was determined from microscopic assay of the inoculum culture with correction for dilution.

Figure 4 provides visual confirmation of *T. ferrooxidans'* release from inhibition by heterotrophic bacteria. Here the inhibitor is an unidentified substance in conventional agar (No. 11849 granulated agar, BBL Microbiology Systems, Cockeysville, MD. 21030). Only at points where *A. cryptum* or *T. acidophilus* grow by consuming organic impurities in the agar can *T. ferrooxidans* grow. Ferric hydroxysulfate, the product of Fe^{2+} oxidation by *T. ferrooxidans*, appears at the foci of intense Fe^{2+} oxidization; these rust-red spots have enlarged to several mm in diameter in 2 weeks. Transplants from the six spots in the upper segment of the dish (labeled 44het), mixed cultures of 44het and AP-19, are identical to the alleged "mixotrophic" *T. ferrooxidans* strain AP-44 (80) from which the strict heterotroph, 44het, was derived (Table 4). Other strains of *T. ferrooxidans* (also *L. ferrooxidans*) respond identically on agar gels with the heterotrophs. Heat-killed heterotrophs do not permit *T. ferrooxidans* to grow.

A similar result occurs on agarose gels containing glucose (Figure 5). The result is more dramatic and occurs in only 2–5 days because glucose is less inhibitory to *T. ferrooxidans* and is a better nutrient for the heterotrophs than the impurities in agar. Eventually, the growth of *T. ferrooxidans* covers completely the surface of the plate because the thousands of *T. ferrooxidans* cells initially spread over it, in aggregate, overcome glucose inhibition. But they grow earliest at the points where the heterotrophs were pricked into the gel and consume the glucose at these points.

It is possible to mistake the foci of intense iron-oxidization for clones of *T. ferrooxidans*. Thus, there have been reports of single-colony isolations of *T. ferrooxidans* that subsequent tests have proven, unequivocally, to be mixed cultures. As already emphasized, any isolate believed able to utilize Fe^{2+} and glucose as sources of energy should be held suspect until the Fe^{2+}-grown and

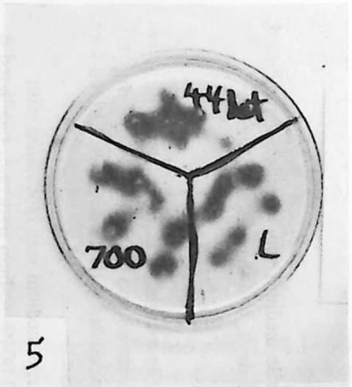


Figure 5 The 9-cm Petri dish contains MS plus 0.5% $FeSO_4 \cdot 7H_2O$ and 0.2% glucose, pH 3, gelled with 0.4% agarose (Type I, No. A-6013; Sigma Chem. Co., St. Louis, MO. 63178). Both autotroph and heterotroph coexist at the foci of the dark circular patches of ferric hydroxysulfate. Legend is the same as given for Figure 4.

the glucose-grown cells are shown to have identical mol% G+C, and also are shown to possess suitably high DNA homology.

Interaction between autotroph and heterotroph can be examined directly. In Figure 6, *T. ferrooxidans* was spread over half of the gel, the upper half as seen in the photograph. Sterile MS-Fe^{2+} medium was spread over the lower half. Five μl MS-glucose culture (10^6 cells) of *T. acidophilus* were deposited at a point on the surface of both halves. It forms buttons of growth at both sites, consuming glucose. On the upper half, *T. ferrooxidans* slowly overcomes inhibition by glucose and forms a flush of growth, seen as a golden discoloration where it was seeded. But near the growth of *T. acidophilus* where glucose is being consumed, *T. ferrooxidans* grows fastest, and, accordingly, it is here that Fe^{2+} oxidation first appears, eventually to form a dark-red halo around *T. acidophilus*.

Cells may be removed from the different zones as the gel is examined under the dissecting microscope. Transplants from the dark halo are autotrophic *T. ferrooxidans*, unable to utilize glucose, and with DNA containing 57 mol% G+C. Transplants from the white button, if care is taken not to dislodge *T. ferrooxidans* cells growing beneath it, are pure *T. acidophilus*, which can grow with glucose but not with Fe^{2+}, and provide DNA that contains 64 mol% G+C. Eventually the button of growth itself becomes discolored, due to admixture of the two species and the development of red ferric hydroxysulfate throughout the area.

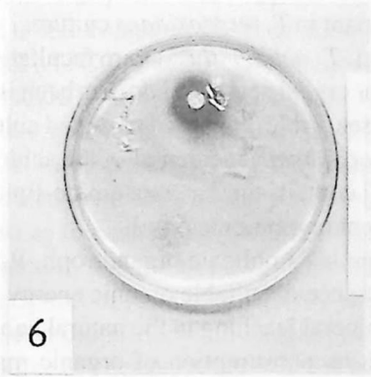


Figure 6 Same medium as in Figure 5. Half of the gel, the upper half as seen in this photograph, received 10^8 cells (0.5 ml culture) of *T. ferrooxidans* DSM 583. The lower half received simply 0.5 ml of sterile culture medium. Five μl (10^6 cells) of *T. acidophilus* DSM 700 culture were deposited in the middle of each half. Consuming glucose, *T. acidophilus* forms large colonies. At the periphery of the colony in the upper half, autotrophic *T. ferrooxidans* grows fast, being released early from glucose inhibition, forming therefore a dark halo of ferric hydroxysulfate around the colony. Further from the colony, cells of *T. ferrooxidans* only slowly overcome inhibition by glucose, eventually to form a pale golden flush of color barely discernible in the photograph. On the lower half of the gel, where no cells of *T. ferrooxidans* were deposited, no oxidation of Fe^{2+} occurs.

In the natural habitat we may expect the heterotrophic acidophiles to be exerting a positive effect on mineral leaching.

ACIDOPHILES THAT GROW AS HETEROTROPHS

Acidiphilium cryptum *and* Thiobacillus acidophilus

Acidophilic heterotrophs were enumerated at ~ 10^3–10^4 g^{-1} coal refuse by Belly & Brock (4), and they were detected in coal mine drainage by Manning (53). But these bacteria were not studied systematically, nor were they assigned a role in their habitat or an activity in relation to *T. ferrooxidans*.

Acidophilic heterotrophs were systematically studied by the author (32). They were derived directly from coal strip-mine refuse and as contaminants in *T. ferrooxidans* cultures. Independently, Wichlacz & Unz (91) isolated them from coal mine drainage. The latter investigators have most extensively monitored such environments, and they have described a variety of biotypes that await species names. In mineral environments, organic substrate may be provided in water drainage, cell material released from autotrophic microorganisms, and perhaps organic substances in association with the metallic sulfides and other minerals.

The Type Species, *Acidiphilium cryptum,* was isolated directly from coal refuse (27) and also as contaminant in cultures of *T. ferrooxidans* from coal mine drainage and from a copper ore leaching site. It is the most common heterotrophic contaminant in *T. ferrooxidans* cultures, with the possible exception of eukaryotic fungi. *T. acidophilus,* a rare facultative lithotroph capable of utilizing either sulfur or organic compounds, has been isolated as a contaminant with *T. ferrooxidans* (see Table 3 for a list of mixed cultures that were formerly presumed pure *T. ferrooxidans*.) Incidentally, the acidophilic heterotrophs and acidophilic Thiobacilli contain similar, rare amino-lipid in their membrane (T. A. Langworthy, personal communication).

Although *A. cryptum* is an obligate organotroph, it is capable of gratuitous sulfur oxidation in presence of suitable organic energy source (29). Therefore, it may play a part in mineral leaching in the natural habitat. At least an indirect role is likely, through its consumption of organic material inhibitory to the autotrophs. (see Table 5 and Figure 4, 5, and 6.) However, *A. cryptum* itself is inhibited by organic substances. Although trypticase and yeast extract in the range of 0.01% to 0.05% may increase growth rate and cell yield, concentrations in the range of 1.0%—customary in conventional bacteriological media—are inhibitory (27, 32). Motility is inhibited prior to loss of viability. Strain Lhet2 in MS containing 0.1% glucose and 0.005% yeast extract[4] is highly

[4]Dehydrated, No. 11929; purchased from BBL Microbiology Systems, Cockeysville, MD. 21030

motile, but when yeast extract concentration is raised to 0.02%, motility is diminished.

Unusual Motility by Acidiphilium Cryptum *Strain Lhet2*

A. cryptum Lhet2 (ATCC 33463) exhibits striking behavior when viewed in a Petroff-Hausser bacteria chamber with the phase-contrast microscope. Young cells (from liquid MS containing 0.1% glucose and 0.005% yeast extract) are actively motile. They swim in circles. As time passes, the diameters of these circles decrease to such an extent that, after a minute, many individual cells may be kept in view without moving the field of vision. All cells swim in the same direction! The cells are sticky, possibly due to the pili, which are seen in electronmicrographs. When a cell collides with the underside of the cover-glass it may adhere thereto. But it continues movement, spinning about the point of contact with the glass, always in the same direction as the cells that swim free in the liquid. On occasion, several cells change their direction—say, from counter-clockwise to clockwise. Then all cells, spinners and swimmers, accept the new direction as if on signal. Motility has not been closely examined in other strains of *A. cryptum*. Flagella have been demonstrated in strain Lhet2 in electronmicrographs (27).

Comparisons Between Acidiphilium Cryptum *Type Strain and Other Heterotrophs*

Table 6 describes strains of *Acidiphilium* isolated by Wichlacz & Unz (personal communication; 91, 92), and compares them to the Type Strain of the Type Species *A. cryptum* Lhet2 (ATCC 33463). The six strains are representatives of 37 strains extensively studied by Wichlacz & Unz and which had been selected by them from a huge collection of heterotrophic acidophiles isolated over a long period of monitoring coal mine drainage in Pennsylvania. My original definition of the genus (27), based on a study of seven strains, has been extended by Wichlacz & Unz (92) so as to encompass the great variety of chemoorganotrophs they have encountered. I am grateful to them for allowing me here to anticipate the new description of *Acidiphilium* they will propose.

Acidiphilium comprises mesophilic, gram-negative, aerobic rods that cannot utilize acetate as sole source of energy, but can utilize citrate and other organic compounds as energy sources. They grow with 0.1% glucose plus 0.01% yeast extract in MS, pH 3. Yet these rods are inhibited by peptones and tissue extracts at concentrations commonly employed in dehydrated media and which serve well the nutritional needs of "conventional" heterotrophs such as *Pseudomonas*. Therefore, they grow in these media only if the media are diluted.

Although sulfur and Fe^{2+} may exist in their natural habitat, in the laboratory they cannot utilize these substances solely as energy for growth. They grow at pH 2.6, some strains grow at pH 2.0, but none grow above pH 6. They are usually, but not always, motile. Glucose catabolism is by a combination of the

Table 6 Comparisons between strains of *Acidiphilium*[a]

	Group 1		Group 2		Group 2_A		*A. cryptum*
	Strain KLB	Strain OP	Strain PW1	Strain PW2	Strain AWB	Strain BBW	Strain Lhet2
Mol% G+C	67	63	65	65	65	65	69±1
Size (μm) in citrate	2.4×0.6	2.0×0.6	1.9×0.8	1.4×0.8	2.0×0.7	1.6×0.6	1.3×0.5
Pigmentation	+	+	−	−	−	−	±
Urea hydrolyzed	−	−	+	+	−	−	
H_2S from cysteine	−	−	−	−	+	+	−
Growth on citrate inhibited by acetate	+	+	−	−	−	−	+
Utilized as sole source of energy							
glucose	−	−	+	+	+	+	+
glycerol	−	−	+	+	+	+	+
lactose	−	−	+	+	+	+	+
glutamate	−	−	+	+	+	+	−
succinate	−	−	+	+	+	+	
malate	−	+	+	+	+	+	
fumarate	−	−	+	+	+	+	
α-keto-glutarate	−	+	+	+	−	−	
ethanol	+	−	+	+	+	+	

[a] All strains utilize citrate as sole source of energy, but none utilize acetate. All strains grow in mineral salts containing 0.1% glucose if a trace of yeast extract (autolysate) is added. Compiled from Wichlacz & Unz (personal communication; 91, 92). DNA homology between *A. cryptum* strain Lhet2 and the other strains examined (PW2, AWB, OP, KLB) is <20%.

Entner-Doudoroff and the pentose phosphate pathways (75). The content of their DNA varies between 63 and 70 mol% G+C. DNA homology between *A. cryptum* ATCC 33463, the Type Strain of the Type Species, and the few other strains tested (PW2, AWB, OP, KLB) is less than 20%. Binomials have not been assigned to all biotypes. D. B. Johnson (37, 38) has encountered several biotypes of acidophilic heterotrophs in "acid streamers" from pyrite mines in Wales and also as contaminants in *T. ferrooxidans* cultures. And heterotrophic bacteria were encountered by DiSpirito et al (18) in uranium ore leaching sites. Acidophilic heterotrophs, undoubtedly, are widespread in acidic mineral environments.

CONCLUDING REMARKS

Numerous strains bearing the binomial *Thiobacillus thiooxidans* should be examined to determine the degree of phenotypic divergence between them.

Then new, related species can be rationally defined. Is there, at present, utility in assigning a new binomial to a single isolate that, itself, is compared to but one strain of an imperfectly delimited *T. thiooxidans?* Fortunately, conservatism has prevailed in the nomenclature of the acidophilic iron-oxidizing bacteria represented by the binomial *Thiobacillus ferrooxidans* despite the fact, or because of it, that a much greater number of pedigreed strains have been examined. If there is too little conservatism in the former example, there may be too much of it in the latter.

Table 3 compares the four taxonomic groups of acidophilic iron-oxidizers. Three of the four do not meet the definition of the genus *Thiobacillus:* they cannot be demonstrated as capable of utilizing sulfur as sole source of energy. If we consider them simply "aberrant," having lost their ability to use sulfur, then we may consider *T. thiooxidans* "aberrant," an "aberrant" *T. ferrooxidans* that has lost its ability to utilize iron. *Leptospirillum ferrooxidans* is as legitimate a species as *Thiobacillus ferrooxidans*. It has zero DNA homology with all strains of *T. ferrooxidans;* it has a lower mol% G+C than any of them; it has distinctive morphology; and it has distinctive motility. Strains of taxonomic group IV also differ from *T. ferrooxidans* in phenotype and in mol% G+C. Though presently unnamed, they also may be considered a distinct genus. If only out of concern for determinative matters, iron-oxidizers must not be irretrievably tied to sulfur-oxidizers in taxonomic keys.

Through an historical accident, the first iron-oxidizing acidophile to be discovered happened also to be sulfur-oxidizer. It was appropriately named *Thiobacillus ferrooxidans*. (If stability in nomenclature is to be gained through a priority rule, names will follow closely historical events.) Suppose, however, that iron-oxidizers had been discovered before sulfur-oxidizers and as a consequence had been named *Ferrobacillus*. A sulfur-oxidizing biotype within this genus becomes *Ferrobacillus thiooxidans*. This is the same biotype as contemporary *Thiobacillus ferrooxidans*. Upon discovery of a sulfur-oxidizing acidophile that did not utilize iron, equivalent in biotype to our *Thiobacillus thiooxidans,* a colleague in this hypothetical situation would experience a problem equivalent to ours with strain m-1 (Table 3). In his taxonomy, however, strain m-1 would be secure as *Ferrobacillus ferrooxidans*. Are we to allow logic in systematics to be overcome simply by a prejudice derived from the *sequence* of discovery?

A radical break with past classification may be necessary when lines-of-descent are established through ribosomal RNA base sequencing underway elsewhere. For the present, the operative classification in Table 3 has utility. There is precedent for this kind of arrangement. Other physiological families are the "Acetic Acid Bacteria" and the "Lactic Acid Bacteria." Another advantage in viewing all iron-oxidizers together in an informal "family" is that new associations may come to mind. Is there a biochemical "connection" between catabolism of iron and catabolism of sulfur to parallel our taxonomic

quandry derived through an historical accident? Is the membranous (respiratory) fine-structure of all these bacteria identical? Is the generation of energy from Fe^{2+} precisely the same in each group?

The demonstration that "glucose-utilizing" *T. ferrooxidans* are usually mixtures of two species, an autotroph and heterotroph (Table 4), provides an exciting prospect. The contaminant (the heterotroph) is in association with the autotroph in the natural habitat and its role there awaits definition. In relatively small populations compared to the heterotrophs that inhabit organic niches, these acidophiles nevertheless may represent a surprising variety of biotypes. They await detection with suitable enrichment-culture techniques. That *Acidiphilium cryptum* (strain Lhet2) at least indirectly aids acidophilic Thiobacilli in mineral leaching there can be little doubt, although this view has yet to be substantiated in the natural habitat.

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Literature Cited

1. Arkesteyn, G. J. M. W., de Bont, J. A. M. 1980. *Can. J. Microbiol.* 26:1057–65
2. Balashova, V. V., Vedenina, I. Ya., Markosyan, G. E., Zavarzin, G. A. 1974. *Microbiol.* 43:581–85
2a. Barros, M. E. C., Rawlings, E. D., Woods, D. R. 1984. *Appl. Environ. Microbiol.* 47: in press
3. Beck, J. V. 1967. *Biotech. Bioeng.* 9:487–97
4. Belly, R. T., Brock, T. D. 1974. *J. Bacteriol.* 117:726–32
5. Bigger, J. W., Nelson, J. H. 1941. *J. Path. Bacteriol.* 53:189–206
6. Borichewski, R. M., Umbreit, W. W. 1966. *Arch. Biochem. Biophys.* 116:97–102
7. Brierley, C. L. 1978. See Ref. 61, pp. 345–63
8. Brierley, J. A., Lockwood, S. J. 1977. *FEMS Microbiol. Lett.* 2:163–65
9. Brierley, J. A., Norris, P. R., Kelly, D. P., Le Roux, N. W. 1978. Eur. *J. Appl. Microbiol. Biotechnol.* 5:291–99
10. Brierley, J. A., Lockwood, S. J., Kelly, D. P., Norris, P. R. 1979. *79th Ann. Meet. Am. Soc. Microbiol. Los Angeles Abstr. 17*
10a. Brierly, J. A., et al, eds. 1984. *Planetary Ecology*. New York: van Nostrand Reinhold. In press
11. Broda, P. 1979. *Plasmids*. San Francisco: Freeman. 197 pp.
11a. Bryant, R. D., McGroarty, K. M., Costerton, J. W., Laishley, E. J. 1983. *Can. J. Microbiol.* 29:1159–70
12. Chandra, D., Roy, P., Mishra, A. K., Chakrabarti, J. N., Trasad, N. K., et al. 1980. *Fuel* 59:249–52
13. Colmer, A. R., Temple, K. L., Hinkle, M. E. 1950. *J. Bacteriol.* 59:317–28

14. Curtin, M. E. 1983. *Biotechnol.* 1:228–35
15. Davis, R. E. 1958. *J. Amer. Chem. Soc.* 80:3565–69
16. DiSpirito, A. A., Tuovinen, O. H. 1982. *Arch. Microbiol.* 133:28–32
17. DiSpirito, A. A., Tuovinen, O. H. 1982. *Arch. Microbiol.* 133:33–37
18. DiSpirito, A. A., Silver, M., Voss, L., Tuovinen, O. H. 1982. *Appl. Environ. Microbiol.* 43:1196–200
19. Dugan, P. R., Apel, W. A. 1978. See Ref. 61, pp. 223–50
20. Ebner, H. G. 1978. See Ref. 61, pp. 195–206
21. Gottschal, J. C., Kuenen, J. G. 1980. *FEMS Microbiol. Lett.* 7:241–47
22. Froudev, S. N., Genchev, F. N., Gaidarjiev, S. S. 1978. See Ref. 61, pp. 253–74
23. Guay, R., Silver, M. 1975. *Can. J. Microbiol.* 21:281–88
24. Guay, R., Silver, M., Torma, A. E. 1976. *Can. J. Microbiol.* 35:62–67
25. Haff, L. V. 1970. In *The Analytical Chemistry of Sulfur and Its Compounds*, ed. J. H. Karchmer, 1:183–283. New York: Wiley. 534 pp.
26. Harrison, A. P. Jr. 1978. *Appl. Environ. Microbiol.* 36:861–69
27. Harrison, A. P. Jr. 1981. *Int. J. Syst. Bacteriol.* 31:327–32
28. Harrison, A. P. Jr. 1982. *Arch. Microbiol.* 131:68–76
29. Harrison, A. P. Jr. 1983. *Int. J. Syst. Bacteriol.* 33:211–17
30. Harrison, A. P. Jr. 1983. *Abstr. Third Int. Symp. Microbial Ecology*, Mich. State Univ., p. 80
31. Harrison, A. P. Jr. 1983. *VI Int. Symp. Environ. Biogeochem.*, Abstr. 5.14, p. 52
32. Harrison, A. P. Jr., Jarvis, B. W., Johnson, J. L. 1980. *J. Bacteriol.* 143:448–54
33. Harrison, A. P. Jr., Lawrence, F. R. 1963. *J. Bacteriol.* 85:742–50
34. Hoffman, M. R., Faust, B. C., Panda, F. A., Koo, H. H., Tsuchiya, M. 1981. *Appl. Environ. Microbiol.* 42:259–71
35. Hutchinson, M., Johnstone, K. I., White, D. 1969. *J. Gen. Microbiol.* 57:397–410
36. Jackson, J. F., Moriarity, D. J. W., Nicholas, D. J. D. 1968. *J. Gen. Microbiol.* 53:5360
37. Johnson, D. B. 1983. *VI Int. Symp. Environ. Biogeochem.* Abstr. 5.12, p. 50
38. Johnson, D. B., Kelso, W. I. 1983. *J. Gen. Microbiol.* 129:2969–72
39. Katayama-Fujimura, Y., Tsuzaki, N., Kuraishi, H. 1982. *J. Gen. Microbiol.* 128:1599–611
40. Kelly, D. P. 1971. *Ann. Rev. Microbiol.* 25:177–210
41. Kelly, D. P., Tuovinen, O. H. 1972. *Int. J. Syst. Bacteriol.* 22:170–72
41a. van Iterson, G. Jr., den Dooren de Jong, L. E., Kluyver, A. J. (1940) 1983. *Martinus Willem Beijerinck, His Life and His Work*, pp. 146–47. Madison, Wis: Science Tech. 181 pp. Reprint
42. Kuenen, J. G., Tuovinen, O. H. 1981. In *The Prokaryotes*, ed. M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegel, pp. 1023–36. New York: Springer
43. Kuenen, J. G., Beudeker, R. F. 1982. *Phil. Trans. Roy. Soc. Lond. B* 298: 473–97
44. Lazaroff, N., Sigal, W., Wasserman, A. 1982. *Appl. Environ. Microbiol.* 43:924–38
45. Leathen, W. W., Braley, S. A., McIntyre, L. D. 1953. *Appl. Microbiol.* 1:65–68
46. Leathen, W. W., Kinsel, N. A., Braley, S. A. 1956. *J. Bacteriol.* 72:700–4
47. Le Roux, N. W., Wakerley, D. S., Sunt, S. D. 1977. *J. Gen. Microbiol.* 100:197–201
48. Lodge, R. M., Hinshelwood, C. N. 1943. *J. Chem. Soc.* 1:213–19
49. London, J. 1963. *Arch. Microbiol.* 46:329–37
50. Lundgren, D. G., Anderson, K. J., Remsen, C. C., Mahoney, R. P. 1964. *Dev. Indust. Microbiol.* 6:250–59
51. Lyons, D., Nickless, G. 1968. In *Inorganic Sulfur Chemistry*, ed. G. Nickless, pp. 509–33. New York: Elsevier. 770 pp.
52. Mackintosh, M. E. 1978. *J. Gen. Microbiol.* 105:215–18
53. Manning, H. L. 1975. *Appl. Environ. Microbiol.* 30:1010–16
54. Markosyan, G. E. 1972. *Biol. Zh. Armenii* 25–26
55. Markosyan, G. E. 1973. *Dokl. Akad. Nauk. SSSR* 211:1205–8
56. Martin, P. A. W., Dugan, P. R., Tuovinen, O. H. 1981. *Can. J. Microbiol.* 27:850–53
57. Mishra, A. K., Roy, P., Mahapatra, S. S. R. 1983. *Curr. Microbiol.* 8:147–52
58. Matin, A. 1978. *Ann. Rev. Microbiol.* 32:433–69
59. Deleted in proof
60. Murr, L. E., Brierley, J. A. 1978. See Ref. 61, pp. 491–520
61. Murr, L. E., Torma, A. E., Brierley, J. A. 1978. *Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena*. New York: Academic. 526 pp.
62. Norris, P. R., Brierley, J. A., Kelly, D.

P. 1980. *FEMS Microbiol. Lett.* 7:119–22
63. Olson, G. J., Iverson, W. P., Brunckman, F. E. 1981. *Curr. Microbiol.* 5:115–18
64. Parker, C. D. 1945. *Australian J. Exptl. Biol. Med. Sci.* 23:81–90
65. Peck, H. D. 1968. *Ann. Rev. Microbiol.* 22:489–518
65a. Perez, R. C., Matin, A. 1980. *J. Bacteriol.* 142:633–38
66. Pol'kin, S. I., Yudina, I. N., Panin, V. V., Kostyukova, O. N., Korostyschevskii, N. B. 1980. *Gidrometallurgiya Zolota M.* 67–71
67. Postgate, J. R., Hunter, J. R. 1962. *J. Gen. Microbiol.* 29:233–63
68. Reynolds, D. M., Laishley, E. J., Costerton, J. W. 1981. *Can. J. Microbiol.* 27:151–61
69. Rittenberg, S. C. 1969. *Adv. Microbiol. Physiol.* 3:159–96
70. Sand, W., Bock, E. 1983. *6th Int. Symp. Environ. Biogeochem.* Abstr. 4.2, p. 41
71. Schaeffer, W. I., Umbreit, W. W. 1963. *J. Bacteriol.* 85:492–93
72. Schmidt, M. 1972. In *Sulfur in Organic and Inorganic Chemistry,* ed. A. Senning, 2:71–112. New York: Dekker. 360 pp.
73. Shafia, F., Brinson, K. R., Heizman, M. W., Brady, J. M. 1972. *J. Bacteriol.* 111:56–65
74. Shehata, T. E., Marr, A. G. 1971. *J. Bacteriol.* 107:210–16
75. Shuttleworth, K. L. 1984. *Glucose catabolism in strains of acidophilic heterotrophic bacteria.* PhD dissertation. Pa. State Univ., University Park. 64 pp.
76. Silverman, M. P. 1967. *J. Bacteriol.* 94:1046–51
77. Sokolova, G. A., Kravaiko, G. I. 1964. *Izdatel'stvo Nauka,* Moscow. 283 pp.
78. Starkey, R. L. 1925. *J. Bacteriol.* 10:135–61
79. Starkey, R. L. 1935. *Soil Sci.* 39:197–219
80. Sugio, T., Kudo, S., Tano, T., Imai, K. 1982. *J. Bacteriol.* 150:1109–14
81. Taylor, B. F., Hoare, D. S. 1969. *J. Bacteriol.* 100:487–79
82. Temple, K. L., Colmer, A. R. 1951. *J. Bacteriol.* 62:605–11
83. Trudinger, P. A. 1967. *Rev. Pure Appl. Chem.* 17:1–24
84. Tuovinen, O. H., Kelly, D. P. 1978. See Ref. 61, pp. 61–81
85. Tuovinen, O. H., Niemela, S. I., Gyllenberg, H. G. 1971. *Antonie van Leeuwenhoek* 37:489–96
86. Tuttle, J. H., Dugan, P. R., Apel, W. A. 1977. *Appl. Environ. Microbiol.* 33: 459–69
87. Vishniac, W. V. 1974. In *Bergey's Manual of Determinative Bacteriology,* ed. R. E. Buchanan, N. E. Gibbons. Baltimore: Williams & Wilkins. 1246 pp.
88. Vishniac, W. V., Santer, M. 1957. *Bacteriol. Rev.* 21:195–213
89. Waksman, S. A., Joffe, J. S. 1921. *Science* LIII:216
90. Waksman, S. A., Joffe, J. S. 1922. *J. Bacteriol.* 7:239–56
91. Wichlacz, P. L., Unz, R. E. 1981. *Appl. Environ. Microbiol.* 41:1254–61
92. Wichlacz, P. L., Unz, R. E. 1982. *XIII Int. Cong. Microbiol.* Abstr. P11.1, p. 29
93. Wieringa, K. T. 1966. *J. Microbiol. Serol.* 32:183–86
94. Zaiser, E. M., LaMer, V. K. 1948. *J. Colloid Science* 3:571–98
95. Zavarzin, G. A. 1972. *Microbiol.* 41: 323–24

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BACTEROIDES OF THE HUMAN LOWER INTESTINAL TRACT

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INTRODUCTION

The human colon contains a numerous and complex microbial flora. Although there are at least 400 distinct species of bacteria, five genera account for approximately 70% of the bacteria that can be cultivated by current techniques (33, 63). *Bacteroides* is one of these five numerically predominant genera and

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accounts for approximately 30% of all fecal isolates. The most numerous *Bacteroides* species in the colon are *Bacteroides vulgatus*, *Bacteroides distasonis*, and *Bacteroides thetaiotaomicron* (10^{10} per g dry weight of feces). Other species such as *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides eggerthii*, *B. uniformis*, and *Bacteroides* "B5–21" are less numerous but are still present in high concentrations (10^9 per g dry weight of feces).

Bacteroides undoubtedly play an important role in the colonic ecosystem. In addition, some colonic *Bacteroides* are opportunistic pathogens. *B. fragilis*, and to a lesser extent other colonic *Bacteroides* such as *B. distasonis*, *B. thetaiotaomicron*, and *B. ovatus*, can cause a variety of infections, including blood stream infections as well as abscesses of the brain, lung, or abdominal cavity (3, 24, 69). Treatment of these infections is complicated by the fact that colonic *Bacteroides* are resistant to many antibiotics (4, 110).

The purpose of this review is to survey recent work on the physiology and genetics of human colonic *Bacteroides*. An excellent review of work done prior to 1979 has appeared (54). Accordingly, this review will focus mainly on research done since 1979.

PHYLOGENETIC CLASSIFICATION

Proof that *Bacteroides* are phylogenetically distant from most other gram-negative bacteria has recently been provided by Paster et al (submitted for publication). On the basis of catalogues of 16S ribosomal RNA from a number of *Bacteroides* strains, they have placed *Bacteroides* in a new phylogenetic group within the eubacteria. This group contains human oral and colonic *Bacteroides* species. Some rumen species, such as *Bacteroides ruminicola*, are included, whereas others, such as *Bacteroides succinogenes* and *Bacteroides amylophilus*, are not. *Fusobacterium*, a genus of gram-negative anaerobes that is one of the five numerically predominant genera in the colon, is also a member of this group. The only other bacteria that have been assigned to the same phylogenetic group as *Bacteroides* are cytophages and flavobacteria.

TAXONOMIC CONSIDERATIONS

Within the genus *Bacteroides*, the principal species of colonic *Bacteroides* form a group that is characterized by the ability to ferment a variety of carbohydrates and by a relatively high tolerance for oxygen. Nutritional requirements of most colonic *Bacteroides* species are simple. They grow readily on a defined medium that contains carbohydrate, ammonia, hemin, vitamin B_{12}, a mixture of inorganic salts, and a reducing agent such as cysteine (93, 117). The main fermentation products are acetate, succinate, and propionate (32).

The numerically predominant species of colonic *Bacteroides* are so similar by standard phenotypic tests that they were originally classified as subspecies of *B. fragilis* (63). However, the results of DNA-DNA homology studies have demonstrated that the colonic *Bacteroides* species are genetically distinct from one another (37). It is becoming more apparent that the differences detected at the genetic level are reflected at the phenotypic level if the right criteria are used. For example, cell surface antigens of different *Bacteroides* species are serologically distinct (70, 72, 73). Patterns of polysaccharide utilization also reflect species differences (93). A greater understanding of how colonic *Bacteroides* species resemble and differ from each other should help to determine why certain *Bacteroides* species are present in the colonic ecosystem in much greater numbers than others and why the most common clinical isolate is *B. fragilis* rather than one of the more numerous colonic *Bacteroides* species. Strain-to-strain differences may also be important. Johnson (38) has shown that strains of a particular *Bacteroides* species that were isolated from the same person had higher DNA-DNA homology with each other than strains that were isolated from different people.

There is no simple selective or differential medium for identifying *Bacteroides* species. Antiserum that is specific for *B. fragilis* and several other *Bacteroides* species has recently become available and should prove useful for identification of *B. fragilis* in clinical specimens (34, 49, 70, 72). A cloned fragment of DNA has been used as a species-specific DNA-dNA hybridization probe for identifying *B. thetaiotaomicron* (88). However, neither species-specific DNA fragments nor species-specific antisera have been obtained for enough different species to make these methods useful for ecological studies.

CELL ENVELOPE

Membrane Lipids

Bacteroides membranes have an unusual lipid composition. Approximately 50% of the lipids that are extractable with chloroform-methanol are spingolipids or free ceramides (59, 62, 108). The main ester-linked fatty acids in extractable lipids are the branched-chain fatty acids, anteiso and isopentadecanoic acid (59, 62). Ester-linked straight chain pentadecanoic acid and hexadecanoic acid are also found, but in lesser amounts (59). The amide-linked fatty acids are exclusively 3-hydroxy fatty acids such as 3-hydroxy iso and anteiso-heptadecanoic acid and 3-hydroxy hexadecanoic acid (59). Thus 3-hydroxy fatty acids are not found solely in lipopolysaccharide.

Lipopolysaccharide (LPS)

Early studies of the endotoxicity of *B. fragilis* LPS revealed that it was far less toxic than the classical enteric LPS (42), and it is unlikely that LPS contributes

significantly to the virulence of *B. fragilis* (41). Studies of the composition of *Bacteroides* LPS have confirmed that it differs in several respects from LPS of other gram-negative bacteria. *Bacteroides* LPS has no 3-keto-2-deoxyoctanoic acid or heptose (40). Both 3-hydroxy and nonhydroxy fatty acids are found in *B. fragilis* LPS. The main 3-hydroxy fatty acids are D-3-hydroxy-penta-, hexa-, and heptadecanoic acid, and D-3-hydroxy-5-methylhexadecanoic acid (124). By contrast, the main hydroxy fatty acid in the LPS of enteric bacteria is 3-hydroxy-tetradecanoic acid. A nonhydroxy branched-chain fatty acid, 13-methyl-tetradecanoic acid, is also found in *B. fragilis* LPS. This fatty acid is exclusively ester-linked. With the exception of 3-hydroxyl-5-methyl-hexadecanoic acid, which is exclusively amide linked, the 3-hydroxy fatty acids can be either in ester or amide linkages (124). The main sugars in *B. fragilis* LPS are L-rhamnose, D-galactose, D-glucose and D-glucosamine (45).

Capsule

Kasper and co-workers have identified and characterized a capsular material that is peculiar to *B. fragilis* (39), and they have proposed that this capsular material is an important determinant of virulence (41, 42, 67, 68). This capsular material can be seen in electron micrographs as a thin, ruthenium red–stained layer. The amount of capsular material decreased when clinical isolates were subcultured on some laboratory media (43), but a rich medium supplemented with 10% fetal calf serum gave high yields of capsule (45). Recently, Kasper and coworkers have developed a method for separating capsular material from membranes, cell wall, and LPS (45). This method should contribute significantly to future research in this area by making it possible to differentiate effects due to LPS and other cell components from effects due to capsule. The main components of purified capsular material were L-fucose, D-galactose, DL-quinovasamine, and D-glucosamine (45). Galacturonic acid was also detected but not quantitated. There was some strain-to-strain variation in the amount and number of sugars detected, and capsular material from two different strains was serologically distinct.

B. vulgatus, *B. thetaiotaomicron*, and *B. ovatus* may also produce capsules. A capsule-like layer is clearly visible around these bacteria when they are stained with India ink (1). There was no correlation between the presence of these capsules and ability of the organism to cause abscesses in mice (9). Encapsulated strains of these *Bacteroides* species were resistant to attack by bacteriophages (9, 12). Other than this, little is known about the capsular material of *Bacteroides* species other than *B. fragilis*. In fact, there is some controversy as to whether the extracellular material that is visualized by India ink is a true capsule (cf. discussion in reference 9). It is clearly different from the capsular material described by Kasper and coworkers.

Inner and Outer Membranes

Kasper & Seiler (44) released an "outer membrane complex" from *B. fragilis* by heating cells at 60°C in a salt-EDTA solution and then homogenizing them in a Waring blender. Because of the heating step, it was not possible to check the preparation for inner membrane contamination by using inner membrane enzymes as markers. Poxton & Brown (71, 72) used a similar procedure to compare outer membrane proteins of different *Bacteroides* species. Diedrich & Martin (20) used Triton X-100 extraction to obtain a detergent-insoluble fraction. All of these methods have the drawback that EDTA or Triton X-100 can selectively release some outer membrane components (50, 98). Recently, we have developed a method for isolation of inner and outer membranes of *B. thetaiotaomicron* without exposure to EDTA or detergent (47a). The method involves two sequential sucrose step gradients. Succinate dehydrogenase activity and LPS-associated 3-hydroxy fatty acids were used as markers for the inner and outer membrane, respectively. The densities of the inner and outer membranes were similar to those of other gram-negative bacteria (1.14 and 1.21 g ml^{-1}, respectively). However, the polypeptide profile of the outer membrane was more complex than the profile usually seen in gram-negative bacteria.

PHYSIOLOGY

Carbohydrate Fermentation

Little is known about the transport of sugars by *Bacteroides*. Uptake of glucose and mannose by *B. thetaiotaomicron* appears to be mediated by specific carriers, and there is evidence that uptake does not involve a phosphotransferase-type system (36). Some of the sugar that is taken up is incorporated into a starch-like polysaccharide (36, 51).

Glucose is fermented via the Embden-Meyerhof pathway (54). Acetate is produced via decarboxylation of pyruvate. Succinate and propionate are produced by a series of steps that begins with the carboxylation of phosphoenolpyruvate to form oxaloacetate. The subsequent reduction of fumarate to succinate may be coupled to NADH oxidation by an electron transport system. Propionate results from the decarboxylation of succinate (54).

The relative amounts of acetate, succinate, and propionate that are produced during growth on glucose vary with the generation time (47). At very slow growth rates, the ratio of propionate to acetate or succinate is higher than at rapid growth rates. Recently, Caspari & Macy (17) have shown that *B. fragilis* growing in continuous culture under a nitrogen atmosphere produced a much greater proportion of propionate than cells growing under a carbon dioxide atmosphere. Also, the specific activity of the CO_2-fixing enzyme PEP carboxykinase increased with decreasing concentrations of CO_2. Thus, *B. fragilis*

reacts to carbon dioxide limitation by increasing PEP carboxylation and by recycling CO_2 via decarboxylation of succinate to form propionate. Apparently this is beneficial to the organism, because the growth yield was highest when cells were grown under a N_2 atmosphere. It is usually assumed that the CO_2 concentration in the colon is high because host fluids contain carbonate. However, the actual concentration of CO_2 available to an organism in the colon may vary due to CO_2 utilization by other bacteria. Thus the ability to adjust to varying CO_2 concentration could be beneficial to *B. fragilis* in its natural habitat.

Growth rates of bacteria growing in the colon are probably quite slow due to limited sources of carbon and energy. When *Bacteroides* are grown on monosaccharides in continuous culture, they generally have maintenance coefficients of less than 0.1 g carbohydrate per g bacteria per hr (17, 47). This means that they have relatively high growth yields even at growth rates as slow as 35 hr per generation. This trait may contribute to their ability to compete successfully in the colonic ecosystem.

Catabolism of Polysaccharides

Monosaccharides and disaccharides in the host's diet do not reach the colon in significant amounts because they are absorbed during passage through the small intestine. Thus the main sources of carbohydrate for obligately saccharolytic bacteria such as *Bacteroides* are probably polysaccharides that are not digestible by the host. These polysaccharides include dietary polysaccharides (e.g. plant cell wall polysaccharides) and host products (e.g. mucins and mucopolysaccharides). Nutritional studies have established that over half of the plant cell wall polysaccharides that are ingested by the host are degraded during passage through the colon (115). There is also some evidence that colon bacteria degrade host products to a comparable extent (118). A survey of strains of the numerically predominant species of colon bacteria revealed that virtually all of the strains that were able to ferment polysaccharides were members of the genus *Bacteroides* (93, 94). Cellulose-degrading bacteria and plant cell wall–degrading bacteria that have been isolated from enrichment cultures of human feces have also turned out to be *Bacteroides* (7, 18). Thus it appears likely that *Bacteroides* are responsible for much of the polysaccharide digestion that occurs in the colon.

Growth yields and maintenance coefficients for colonic *Bacteroides* growing in continuous culture on polysaccharides have only been determined for one species, *B. thetaiotaomicron,* and only for two polysaccharides, chondroitin sulfate and arabinogalactan (84, 90). In both cases yields were comparable to yields obtained when bacteria were grown on component monosaccharides. Maintenance coefficients were somewhat higher for growth on polysaccharides than for growth on component monosaccharides, but the bacteria were still able

to grow on polysaccharides at growth rates as slow as those that are probably experienced by the organism in the colon.

Some species of *Bacteroides* such as *B. thetaiotaomicron* can ferment a variety of plant and host polysaccharides. This versatility may be an advantage in the colon, where bacteria are confronted with a constantly changing mixture of dietary polysaccharides. So far, all of the polysaccharide-degrading enzymes of colonic *Bacteroides* that have been studied appear to be regulated. High specific activities of a particular polysaccharidase are detected only when the organism is grown on the appropriate polysaccharide (85, 87, 92). Induction of enzyme synthesis occurs fairly rapidly. For example, when *B. thetaiotaomicron* is exposed to chondroitin sulfate, a host mucopolysaccharide, synthesis of the degradative enzymes begins within 30 min and the maximum specific activity is reached within 2 hr (87). Thus the organism appears to be flexible enough to switch from one substrate to another, although there may be as yet undiscovered conditions in the colon that prevent this from happening.

Induction of the synthesis of enzymes such as chondroitin lyase and α-glucosidase does not occur if a more readily fermentable substrate such as glucose is available (91). Whether this is the same phenomenon as catabolite repression, which it resembles superficially, has not been determined. Repression is not overcome by exogenous cAMP or dibutyryl cAMP (A. Salyers, unpublished observation). This could be due to the organism's inability to take up cAMP or dibutyryl cAMP, although attempts to detect cAMP or adenylate cyclase in *B. thetaiotaomicron* have been unsuccessful (35, 100).

In some cases, the polysaccharide-degrading enzymes of colonic *Bacteroides* are cell-associated rather than extracellular (85, 89, 92). Polysaccharides such as chondroitin sulfate or laminarin that are degraded by cell-associated enzymes are too large to diffuse readily through pores that admit mono- or disaccharides. Yet, the chondroitin sulfate-degrading enzymes of *B. thetaiotaomicron* are not on the cell surface and may be in the periplasm (89). Recently, we have found that there are outer membrane polypeptides that are associated with growth on chondroitin sulfate (47a). The precise function of these polypeptides has not yet been determined.

In nature, most polysaccharides are not free in solution but are bound in complex matrices (e.g. plant cell wall, proteoglycan). In such cases, cell-associated enzymes would be expected to be less efficient than extracellular enzymes. Nevertheless, *B. thetaiotaomicron* is able to digest the chondroitin sulfate that is bound up in proteoglycan nearly as efficiently as it can digest free chondroitin sulfate (48).

There have been several reports of extracellular polysaccharide-degrading enzymes (2, 83, 86, 97). Although cell lysis was not ruled out in most of these studies, it is possible that some polysaccharide-degrading enzymes are secreted.

Only a few of the polysaccharide-degrading enzymes that are produced by colonic *Bacteroides* have been isolated and characterized. Two chondroitinases have been isolated from *B. thetaiotaomicron* (52). These enzymes were similar in size (M_r of 104,000 and 107,000) and in affinity for various mucopolysaccharides. Scudder et al (97) obtained a 7000-fold purification of an endo-ß-galactosidase from *B. fragilis*. The enzyme had an apparent molecular weight of 32,000 and was able to digest the mucopolysaccharide keratan sulfate.

Sialidase (neuraminidase) from *B. fragilis* has been purified by two groups. *B. fragilis* is able to digest several types of mucin (81), and sialidase probably contributes to the breakdown of these very complex molecules. Berg et al (5) found three neuraminidase activities that were separable on an isoelectric focusing gel (pI = 6.8, 7.1, and 7.4). The activity with a pI of 7.1 was purified 542-fold. It had a molecular weight of 92,000, was stimulated by divalent cations, and was inhibited by EDTA. This neuraminidase was able to remove terminal sialic acid residues from bovine submaxillary mucin, fetuin, and porcine gastric mucin. Von Nikolai et al (119) partially purified a sialidase from *B. fragilis* with a molecular weight of 180,000. Activity was not affected by divalent cations or EDTA. The pI was not measured, so it is not clear whether this was one of the neuraminidases detected by Berg and coworkers.

Finally, Berg et al (6) have partially purified six glycosidases from *B. fragilis*, including α-glucosidase; ß-glucosidase, α-galactosidase, ß-galactosidase, ß-N-acetyl glucosamididase, and α-L-fucosidase. Some of these activities were separable into more than one electrophoretically distinct form. These enzymes may contribute to breakdown of mucins and other polysaccharides.

Utilization of Amino Acids and Proteins

Colonic *Bacteroides* cannot use amino acids or proteins as the sole source of carbon and energy, nor do they require amino acids or peptides when they are growing on carbohydrates. For these reasons it is often assumed that *Bacteroides* are unable to use these compounds, except perhaps to take up amino acids and incorporate them directly into protein. Two reports indicate that this view might be too simplistic. Smith & Salyers (104) found that when labeled leucine, isoleucine, or valine was taken up by *B. thetaiotaomicin*, only half of it was incorporated into protein. The remainder was incorporated into membrane lipids, presumably after removal of the amino group. Riepe et al (80) found that a clinical isolate of *B. fragilis* secreted proteolytic activity. The proteolytic activity was produced only during stationary phase and only when bacteria were grown in defined media.

Effect of Oxygen

Although colonic *Bacteroides* will not grow if oxygen is present in the medium, they can survive exposure to oxygen for prolonged periods. Some species of colonic *Bacteroides* produce catalase (123). *B. fragilis* and *B. distasonis* also produce superoxide dismutase (SOD). The *B. fragilis* SOD is an iron-containing enzyme (25). Its synthesis is induced by oxygen, and fully induced cells are more resistant to hyperbaric oxygen than uninduced cells (74). The *B. fragilis* SOD has been purified to apparent homogeneity (26). It is a dimer, composed of two 42,000-dalton monomers. Iron can be removed from the enzyme and the inactive apoprotein can be reconstituted with iron to form active enzyme. Oddly enough the inactive enzyme can also be reconstituted with manganese, although no MnSOD has been found in *B. fragilis*.

Oxygen enhances host cell reactivation of UV-irradiated bacteriophage by *B. fragilis* (103). Oxygen also makes *B. fragilis* more sensitive to UV light (101, 102). Bacteria that were exposed to UV light under anaerobic conditions induced synthesis of three proteins that could be components of some sort of host repair system (96). The effect of oxygen on host cell reactivation and sensitivity to UV light may be due to some effect of oxygen on this repair system.

Production of Mutagens

The hypothesis that colonic bacteria are somehow involved in the etiology of colon cancer has appeared in print so often that it has almost acquired the status of fact. However, there has been little direct evidence either to refute or confirm this hypothesis. Recent findings have reopened the question of the relationship between the colonic flora and colon cancer in an intriguing way. It has been shown that some people excrete a substance that is mutagenic on the Ames test. The incidence of mutagen-excreters in different populations parallels the incidence of colon cancer (21). Van Tassel et al (116) screened forty species of colonic anaerobes and found that only five species, all of them *Bacteroides*, could produce a mutagenic substance. The structure of the mutagen has been deduced from chemical ionization mass spectra (31). It is a simple glyceryl ether lipid. The lipid portion contains 12 carbons and 5 conjugated double bonds. Its metabolic precursor has not been identified, nor has enough of the mutagen been accumulated to permit testing for carcinogenicity. Even if the mutagen proves to have nothing to do with human intestinal disease, it may still point to an as yet undiscovered metabolic capability of *Bacteroides*.

It is interesting to note that *Bacteroides* can also inactivate mutagenic substances. Kinouchi & Ohnishe (46) have separated and characterized four 1-nitropyrene reductases from *B. fragilis*. These enzymes reduced the mutagenicity of several nitro-compounds. At present, it is not clear whether such enzymes actually enable *B. fragilis* to protect its human host from toxic substance. Nor is it clear what role these enzymes play in the cellular metabolism of *B. fragilis*.

Resistance to Antibiotics

Human colonic *Bacteroides* are uniformly resistant to all aminoglycosides. This resistance is probably due to failure to transport aminoglycosides (11). Most colonic *Bacteroides* are resistant to ß-lactam antibiotics (4, 110). *Bacteroides* produce a variety of ß-lactamases (4, 65, 66, 95, 109, 111). Originally these ß-lactamases were thought to be mainly cephalosporinases (4). Recently, Sato and coworkers (95) isolated a ß-lactamase from *B. fragilis* that is more active on penicillins than cephalosporins. Moreover, Cuchural and coworkers (submitted for publication) have found strains of *B. fragilis* that produce ß-lactamases that degrade cefoxitin, a semisynthetic cephalosporin that is not degraded by most ß-lactamases. *Bacteroides* have penicillin-binding proteins in their membranes (10), but nothing is known about the relationship between these proteins and susceptibility to ß-lactam antibiotics. About two thirds of all colonic *Bacteroides* are resistant to tetracycline (4, 110). The mechanism of resistance has not been studied in detail but appears to involve altered transport of the drug (22). A detailed review of research on antibiotic resistance in *Bacteroides* has been published (4).

Most strains of *Bacteroides* are susceptible to chloramphenicol, metronidazole, and clindamycin. Resistance to clindamycin, the drug of choice for treating *Bacteroides* infections, appears to be increasing (4, 110). This increase, and the increase in resistance to tetracycline, may be due in part to conjugal transfer of resistance genes (see below).

GENETICS

Cryptic Plasmids

Many *Bacteroides* strains contain plasmids, and it is not unusual for a single strain to have more than one plasmid (16, 60, 107, 120). Most of the plasmids are 3–7 kb in size, but plasmids as large as 150 kb have been reported. Some plasmids are found in more than one species of *Bacteroides*. This was first shown by Mays & Johnson (60), who used DNA-DNA reassociation to determine the homology between plasmids from 4 strains of *Bacteroides* and plasmids from 23 other plasmid-bearing strains of *Bacteroides*. One plasmid in particular, a 4.4-kb plasmid from *B. uniformis* T1–1, was homologous with plasmids from 15 of the 23 *Bacteroides* strains tested. More recently, Callihan et al (16) used Southern blot analysis and restriction enzyme digestion to show that the small plasmids (<8 kb) that were found in 15 clinical and natural isolates of *Bacteroides* could be assigned to three DNA homology classes. So far no phenotype has been assigned to any of these ubiquitous small plasmids. Traits such as resistance to heavy metals, resistance to antibiotics, or production of bacteriocins are not associated in any obvious way with the presence of these plasmids (120). These small cryptic plasmids that are so frequently seen in *Bacteroides* may be parasites that encode only the functions needed for their own replication. However, it is also possible that these plasmids have some ecological significance.

Conjugal Transfer of Antibiotic Resistance Among Bacteroides

CLINDAMYCIN-ERYTHROMYCIN To date, resistance to clindamycin and erythromycin is the only phenotype that has been convincingly associated with a *Bacteroides* plasmid. Evidence that resistance to clindamycin and erythromycin might be carried on a plasmid was first provided by Privitera et al (75) and by Tally et al (113). Privitera et al (75) demonstrated that a strain of *B. fragilis* could transfer resistance to clindamycin, erythromycin, and streptogramin to another *B. fragilis* strain and that these resistances could be cured en bloc. Welch et al (121) showed that transfer of antibiotic resistance occurred by a conjugation-like process and was associated with transfer of a 41-kb plasmid, called pIP410 by Privitera and coworkers (55) and pBF4 by Welch et al. Transcipients could retransfer the plasmid. The frequency of transfer among isogenic strains was 100–1000-fold higher than among strains of different species (121). Welch & Macrina (122) constructed a partial restriction map of pBF4 and isolated two spontaneous deletions of pBF4 that no longer conferred clindamycin resistance (Figure 1A). From this, they concluded that the gene for clindamycin resistance, or a portion of it, is located somewhere in the *Eco*RI-D fragment of pBF4 (53, 122). Welch & Macrina also examined homoduplexes of pBF4 and found evidence for two sets of inverted repeats (IR1 and IR2 in Figure 1A). Similar inverted repeats have been found in pIP410 by Magot et al (55), although these repeats were not mapped with reference to any of the restriction sites shown in Figure 1A.

pBFTM10, the conjugative clindamycin resistance plasmid described by Tally and coworkers (113, 114), is much smaller than pBF4 (pIP410), i.e. 14.6 kb as compared to 41 kb. A partial restriction map of pBFTM10 is shown in Figure 1B. A deletion, indicated by Δ1 in Figure 1B, is associated with loss of resistance to clindamycin. From conjugation experiments it has been concluded that pBFTM10 carries resistance to clindamycin and erythromycin but not to tetracycline, although tetracycline resistance is sometimes co-transferred. Unlike pBF4, pBFTM10 was transferred at a much higher frequency if the donor was exposed to clindamycin or tetracycline prior to mating. Also in contrast to pBF4, when pBFTM10 was transferred from the *B. fragilis* strain in which it was originally found to another *B. fragilis* strain, the frequency of transfer from the transcipient to an isogenic strain was much lower than the frequency of transfer out of the original strain. Transfer was no longer enhanced by pretreatment of the donor with clindamycin. This raised the question of whether the transfer of pBFTM10 out of the original strain was facilitated by genes in the host strain other than those that are carried on pBFTM10 itself (56).

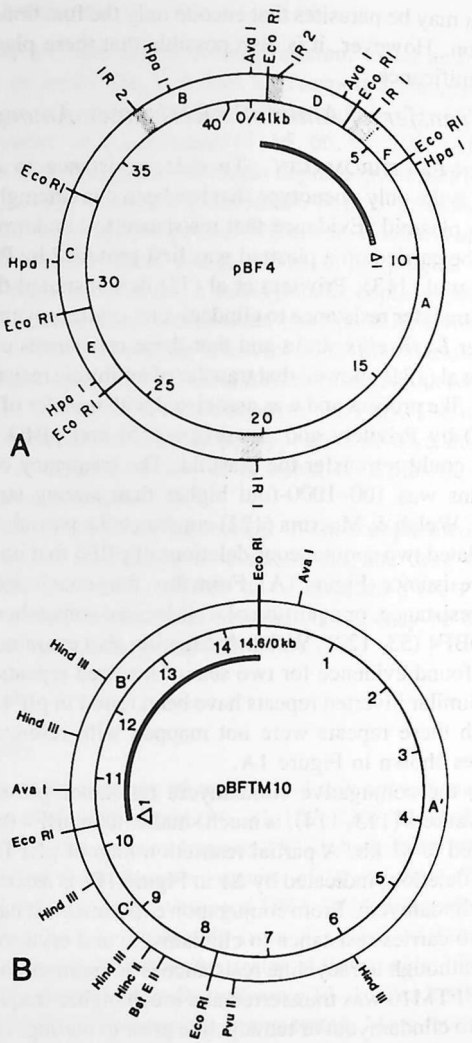


Figure 1 Partial restriction map of the clindamycin resistance plasmids pBF4 (*A*) and pBFTM10 (*B*). Size is given in kb. The deletions (Δ1), indicated by heavy lines, are associated with susceptibility to clindamycin. In the case of pBF4, a smaller deletion has been isolated (Δ2; not shown). This deletion spans the *Eco*RI-D fragment and is also associated with susceptibility to clindamycin (53, 122). Δ2-like deletions were more commonly isolated than Δ1-like deletions (121). The inverted repeat (IR) sequences in pBF4 (shaded areas) were identified by homoduplex analysis (122). No such inverted repeats have been found in pBFTM10 (99). Reprinted with permission from Shimell et al (99).

There is some evidence that pBFTM10 can mobilize small cryptic plasmids (56). Similar mobilization by pBF4 has also been reported (78). Guiney and coworkers (submitted for publication) have found a spontaneous transfer-deficient mutant of *B. fragilis* V479, the strain that harbors pBF4. This transfer-deficient strain still carried pBF4, but the plasmid had a 3-kb insert near the 15-kb mark in the *Eco*RI-A fragment of pBF4 (Figure 1A). Thus at least some of the transfer functions of pBF4 may be located in this region.

Although pBFTM10 is clearly different from pBF4 in size and restriction pattern, the *Eco*RI-D fragment of pBF4 and the *Eco*RI-B' fragment of pBFTM10 cross-hybridize on Southern blots (99). This homology has been interpreted as evidence that similar or identical genes for clindamycin resistance are carried by the two fragments. Neither the cloned *Eco*RI-D fragment from pBF4 nor the cloned *Eco*RI-B' fragment from pBFTM10 confer increased resistance to clindamycin-erythromycin on *E. coli* (99, 112), but this could be due to failure of *E. coli* to synthesize the *Bacteroides* protein or to failure of the *Bacteroides* protein to function properly in *E. coli*. It has been noted that both the B' region of pBFTM10 and the D region of pBF4 are spanned by adjacent *Ava*I-*Eco*RI sites that may be direct repeats (56, 99). If so, this could explain the high frequency of spontaneous deletions in this region.

There are cases in which transfer of clindamycin resistance appears not to involve transfer of a plasmid (29, 58, 61, 112). Chromosomal DNA from these strains contains a region that is homologous with the *Eco*RI-B' fragment of pBFTM10 or with the *Eco*RI-D fragment of pBF4. The clindamycin resistance determinants of these strains could be located on large, low copy plasmids that are difficult to separate from chromosomal DNA. Alternatively, as Tally et al (112) have pointed out, the DNA that codes for clindamycin resistance may be chromosomal, with conjugal transfer occuring via a "conjugal transposon" similar to those that have been associated with transfer of MLS resistance among streptococci (23, 106).

Some recent evidence supports the hypothesis that the clindamycin resistance determinant of pBF4 or pBFTM10 may be on a transposon or on some element that participates in genetic rearrangements. Smith & Macrina (103a) found two plasmids in a clindamycin-resistant strain of *B. ovatus*. One of them, pBI136 (an 81-kb plasmid), was associated with the transfer of clindamycin resistance from this strain to other *Bacteroides*. pBI136 exhibited homology with the *Eco*RI-D fragment of pBF4 and the *Eco*RI-B' fragment of pBFTM10 but not with other portions of these plasmids. The second plasmid, pBI106 (a 46-kb plasmid), was not associated with transfer of clindamycin resistance. However, this plasmid shared extensive homology with all of pBF4 except the *Eco*RI-D region. Smith & Macrina suggest that these two plasmids may be the result of an earlier recombination event involving the pBF4 clindamycin determinant, and they interpret this as evidence that the clindamycin resistance gene is located on a transposon.

Guiney and coworkers (submitted for publication) have discovered a similar case. They isolated a 15-kb plasmid (pCP1) from a clinical strain of *B. thetaiotaomicron*. This plasmid appears to encode clindamycin resistance and exhibits homology with the *Eco*RI-D fragment of pBF4. Because the size and restriction pattern of pCP1 are identical to those of pBFTM10, pCP1 is either identical to pBFTM10 or closely related. Another plasmid, a 10-kb plasmid (pCP2), which was found in a strain of *B. distasonis* isolated from the same infection, did not carry clindamycin resistance. However, pCP2 had extensive homology with regions of pCP1 other than the region that cross-hydridized with the *Eco*RI-D fragment of pBF4, and DNA homologous of the *Eco*RI-D fragment of pBF4 was found in the chromosome of the organism that harbors pCP2.

TETRACYCLINE The *B. fragilis* strain (V479), which harbors the clindamycin resistance plasmid pBF4, is also capable of transferring tetracycline resistance to other *Bacteroides* (105). However, resistance to tetracycline is transferred independently of resistance to clindamycin, and no detectable plasmid is associated with its transfer. Both resistance to tetracycline and transfer frequency of tetracycline resistance are enhanced by pretreatment of the strain with subinhibitory concentrations of tetracycline (76, 105). Conjugal transfer of tetracycline resistance, which is not associated with any detectable plasmid, has now been demonstrated for a number of *Bacteroides* strains (29, 56, 78).

Some *Bacteroides* strains transfer both resistance to clindamycin-erythromycin and resistance to tetracycline in bloc. One such strain was described by Mays et al (61). This strain, originally designated 12256 by the VPI Anaerobe Laboratory and renamed V503 by Mays and coworkers, contains a 3.7-Md plasmid. The plasmid is cotransferred with resistance to tetracycline-clindamycin about 80% of the time but does not carry the resistance determinants. A single *Hind*III restriction fragment of chromosomal DNA from transcipients hybridizes with the *Eco*RI-D fragment of pBF4 but not with pBF4 Δ1 (which does not contain the D fragment). Thus the transmissible clindamycin resistance may be related to the clindamycin determinant on pBF4 and pBFTM10.

Raschtian & Booth (77) reported that *B. fragilis* 12256 (V503) contained a 2-Md plasmid in addition to the 3.7-Md plasmid mentioned above. When they isolated this 2-Md plasmid (pBY2) and used it to transform *E. coli*, the transformants were resistant to both tetracycline and penicillin. This report is controversial. Neither the strain studied by Mays and coworkers (61), nor the same strain obtained by us from the VPI Anaerobe Laboratory contained a 2-Md plasmid or any plasmid that could confer tetracycline resistance on *E. coli*. Moreover, an isolate of strain 12256 that we obtained from Booth no longer contained such a plasmid (A. Salyers, unpublished results). Thus the source of this very interesting small plasmid remains obscure.

Interestingly enough, Guiney and coworkers have discovered that the *Eco*RI-D fragment of pBF4, cloned in vector R300B, contains a tetracycline resistance that is expressed in *E. coli*. (Guiney et al, submitted for publication.) Clearly this resistance is not expressed productively in *Bacteroides* because transfer of pBF4 from one *Bacteroides* strain to another does not make the recipient resistant to tetracycline. Unlike previously studied genes for tetracycline resistance, the resistance determinant from pBF4 is expressed when the *E. coli* strain is grown aerobically, but not when it is grown anaerobically. The tetracycline determinant on pBF4 is not homologous with the tetracycline resistance determinants on R100, RK2, or pBR322 or with the conjugative tetracycline resistance of *B. fragilis* V479.

Rotimi et al (82) reported that resistance to clindamycin, chloramphenicol, and tetracycline was transferred by conjugation from clinical isolates of *B. fragilis* and *B. thetaiotaomicron* to other strains of *Bacteroides*. They claimed that transfer of resistance was associated with transfer of large plasmids (39–62 Md), but no plasmid gels were shown, and it was not clear how many transcipients were surveyed. No further investigations of these strains, either by this group or by other groups, have appeared subsequently.

ß-LACTAM ANTIBIOTICS To date there have only been three reports of transfer of resistance to ß-lactam antibiotics. Tally and coworkers (15, 112) found that a strain of *B. fragilis* (TMP14) that was resistant to ampicillin did not transfer ampicillin resistance to another *B. fragilis* strain. If, however, this strain was first mated with a strain of *B. fragilis* that carried a transferable tetracycline resistance (TM 2000), the progeny that were resistant to both tetracycline and ampicillin were able to transfer ampicillin resistance to other *B. fragilis* strains. Raschtian and coworkers (79) have reported that a clinical isolate of *B. thetaiotaomicron* (UN101) transfered cefoxitin resistance to other *Bacteroides* strains by a conjugation-like process. No plasmid was associated with transfer, and the transcipients were unable to transfer ampicillin resistance to other strains. Sato et al (95) found a clinical isolate of *B. fragilis* that could transfer ampicillin resistance to *B. fragilis* or to *B. vulgates*. In this case, transcipients were able to retransfer the resistances. However, these transcipients had acquired tetracycline resistance along with the ampicillin resistance. Thus, the ampicillin resistance could have been mobilized by the tetracycline resistance determinant. So far, it is not clear whether any of these ß-lactam resistance determinants is self-mobilizing.

Conjugal Transfer Between Bacteroides *and* E. coli

If *Bacteroides* is as distant phylogenetically from *E. coli* as the data of Pastern & Woese indicate, it would seem reasonable to expect that conjugal

transfer between *Bacteroides* and *E. coli* occurs rarely if at all in nature. However, there have been several reports of conjugal transfer of resistance determinants between *Bacteroides* and *E. coli*. In one case, the organism involved has turned out to be not a true *Bacteroides*. Guiney & Davis (27) found a plasmid in *Bacteroides ochraceus* (pGD10), an oral anaerobe, that could be transferred by conjugation from *B. ochraceus* to *E. coli*. This plasmid carried resistance to chloramphenicol, tetracycline, kanamycin, and streptomycin. Recent taxonomic investigations of the strains that were originally named *B. ochraceus* have resulted in their being reclassified as *Capnocytophaga ochraceus* (28). Interestingly, *C. ochraceus* was not able to transfer tetracycline resistance to *B. fragilis* (28).

Guiney & Davis (28) were unable to demonstrate transfer of plasmids such as R100 *drd*1, R64 *drd*11 or RK2 from *E. coli* to *B. fragilis*. Similarly, Del Bene and coworkers (19) screened a number of *E. coli* strains that carried transmissible plasmids and were also unable to demonstrate transfer of any of these plasmids from *E. coli* to *B. fragilis*. By contrast, Burt & Woods (13) claimed that they were able to transfer ampicillin resistance from *E. coli* to *B. fragilis*. Both the *E. coli* strains and the *Bacteroides* strains were isolated from feces. Their criteria for identifying strains as *B. fragilis* were gram stain, anaerobic growth, resistance to kanamycin and vancomycin, and ability to grow in medium containing deoxycholate, rather than the standard criteria for speciation. Thus it is possible that they were working with some organism other than *Bacteroides*. This report, which appeared in 1976, has not been followed up either by this group or by any other researchers.

Three groups have reported transfer of drug resistances from *Bacteroides* to *E. coli* (57, 82, 124). Mancini & Behme (57) found that a clinical isolate of *B. fragilis* could transfer resistance to ampicillin, amoxicillin, cephalothin, tetracycline, minocycline, and chloramphenicol to *E. coli* K12. Rotimi et al (82) reported that clinical isolates of *B. fragilis* and *B. thetaiotaomicron* could transfer both chloramphenicol and tetracycline resistance to *E. coli* K12. One strain of *B. fragilis* transferred tetracycline resistance alone to *E. coli*. A peculiar feature of this transfer was that the frequency of transfer from *B. fragilis* to *E. coli* was the same as the frequency of transfer from *B. fragilis* to *B. fragilis*. There have been no reports of successful transfer to *E. coli* of tetracycline resistances from the conjugative *B. fragilis* strains that have been studied by other groups. We have not been able to demonstrate transfer of tetracycline resistance from either *B. fragilis* V479 or *B. fragilis* 12256 (V503) to *E. coli* (A. Salyers, unpublished results). Unfortunately, there have been no further published reports of work with the strains described by Mancini & Behme (57) or by Rotimi et al (82).

Young & Mayer (125), in a 1979 review, described the conjugal transfer from *B. fragilis* to *E. coli* of DNA that coded for a ß-lactamase. A 3-Md plasmid from the *B. fragilis* strain conferred ampicillin resistance on the *E. coli* strain when it was introduced by transformation. However, when ampicillin resistance was transferred by conjugation from *B. fragilis* to *E. coli*, none of the transcipients contained the 3-Md plasmid. No further description of this work has yet been published. At present, it is not possible to say with certainty whether *Bacteroides* can exchange drug resistances with *E. coli*. Further work needs to be done with the *Bacteroides* isolates that have been reported to conjugate with *E. coli*.

One of the main difficulties involved in demonstrating conjugal transfer between *E. coli* and *Bacteroides* is that many *Bacteroides*, especially *B. fragilis*, can grow in coculture with *E. coli* under aerobic conditions. Apparently, *E. coli* can remove oxygen from the medium rapidly enough to permit *Bacteroides* to grow, even when liquid cultures are shaken vigorously in air. *Bacteroides* can also grow together with *E. coli* in colonies on aerobic plates. For this reason, it is essential that all apparent transconjugants be colony purified more than once and that the resulting cultures be checked for *Bacteroides* contamination by growing them anaerobically and testing for unique *Bacteroides* markers.

Transformation

In 1977, Burt & Woods (14) reported that a strain of *B. thetaiotaomicron* could be transformed with bacteriophage DNA. The method used was a calcium shock method similar to that used for transforming *E. coli*. This work has not been followed up by the group that published it. Recent attempts to use this method, or variations of it, to introduce pBF4 or pBFTM10 into *Bacteroides* have not been successful (J. Smith, unpublished results; A. Salyers, unpublished results). There is one report that *Bacteroides* secrete DNase (83). No further studies of DNase production by *Bacteroides* have been published. Secreted DNase could interfere with transformation.

Bacteriophages and Bacteriocins

A number of *Bacteroides* phages have been isolated from sewage (9). So far, all of these phages have proven to be lytic phages. Neither lysogeny nor transduction has been demonstrated. Oddly enough, although *B. vulgatus* is the most common isolate from feces, no *B. vulgatus* phages have been found. Also, although phages for colonic *Bacteroides* are readily isolated from sewage, no one has yet reported a successful attempt to isolate them directly from human feces.

Bacteriocin production by strains of human colonic *Bacteroides* was first reported by Booth et al (8). A bacteriocin that is produced by *B. uniformis* T1–1 has a M_r of about 300,000. This bacteriocin is extremely stable to heating and to

a wide range of pH values (8). The mechanism of action has not been determined. Mossie et al (64) have described a bacteriocin that is produced by *B. fragilis* and that has a M_r of 13,500–18,700. This bacteriocin appears to inhibit RNA synthesis in susceptible strains. Hayes et al (30) have isolated another bacteriocin from *B. fragilis* that has a M_r of 5,000. Its mode of action has not yet been determined.

CONCLUDING REMARKS

Our understanding of metabolic and genetic capabilities of *Bacteroides* has advanced considerably during the past decade. It is still true that virtually nothing is known about physiology and regulation at the molecular level. Recently, Guiney et al (submitted for publication) constructed a chimeric plasmid (pDP1) that is mobilized from *E. coli* to *B. fragilis* by pRK231. Subsequently, we have constructed a similar chimeric plasmid (pE5-2) that is mobilized from *E. coli* to *Bacteroides* by R751 (N. Shoemaker et al, submitted for publication). The ability to move foreign DNA into *Bacteroides* will now make it possible to manipulate these organisms genetically. But there is still no method for genetic mapping in *Bacteroides*.

Perhaps the greatest challenge in future *Bacteroides* research, however, is to devise methods for measuring specific components or metabolic activities of these organisms in colon contents or clinical specimens. To date, virtually all of our ideas about the possible role of *Bacteroides* in the colonic ecosystem or in infections are based on studies of pure cultures growing under laboratory conditions. The hypotheses that arise from such laboratory studies need to be tested by direct measurements of the actual characteristics or activities of the organisms when they are growing in their natural habitat.

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Literature Cited

1. Babb, J. L., Cummins, C. S. 1978. *Infect. Immunity* 19:1088–91
2. Balascio, J. R., Palmer, J. K., Salyers, A. A. 1982. *J. Food Biochem.* 5:271–82
3. Bartlett, J. G. 1983. *Rev. Infect. Dis.* 5:235–45
4. Bawdon, R. E., Crane, L. R., Palchardhuri, S. 1982. *Rev. Infect. Dis.* 4:1075–95
5. Berg, J. O., Linqvist, L., Anderson, G., Nord, C. E. 1983. *Appl. Environ. Microbiol.* 46:75–80
6. Berg, J. O., Linqvist, L., Nord, C. E. 1980. *Appl. Environ. Microbiol.* 40:40–47
7. Betian, H. G., Linehan, B. A., Bryant, M. P., Holdeman, L. V. 1977. *Appl. Environ. Microbiol.* 33:1009–10
8. Booth, S. J., Johnson, T. L., Wilkins, T. D. 1977. *Antimicrob. Agents Chemother.* 11:718–24
9. Booth, S. J., Van Tassell, R. L., Johnson, J. L., Wilkins, T. D. 1979. *Rev. Infect. Dis.* 1:325–36
10. Botta, G. A., Privitera, G., Menozzi, M. G. 1983. *J. Antimicrob. Chemother.* 11:325–31
11. Bryan, L. E., Kowand, S. K., Van Den Elzen, H. M. 1979. *Antimicrob. Agents Chemother.* 15:7–13
12. Burt, S., Meldrum, S., Woods, D. R., Jones, D. T. 1978. *Appl. Environ. Microbiol.* 35:439–43
13. Burt, S. J., Woods, D. R. 1976. *J. Gen. Microbiol.* 93:405–9
14. Burt, S. J., Woods, D. R. 1977. *J. Gen. Microbiol.* 103:181–87
15. Butler, T., Tally, F. P., Gorbach, S. L., Malamy, M. 1980. *Clin. Res.* 28:35
16. Callihan, D. R., Young, F. E., Clark, V. L. 1983. *Plasmid* 9:17–30
17. Caspari, D., Macy, J. M. 1983. *Arch. Microbiol.* 135:16–24
18. Dekker, J., Palmer, J. K. 1981. *J. Agric. Fd. Chem.* 29:480–84
19. Del Bene, V. E., Rogers, M., Farrar, W. E. 1976. *J. Gen. Microbiol.* 92:384–90
20. Diedrich, D. L., Martin, A. E. 1981. *Curr. Microbiol.* 6:85–88
21. Ehrich, M., Aswell, J. G., Van Tassell, R. L., Wilkins, T. D. 1979. *Mutat. Res.* 64:231–40
22. Fayolle, F., Privitera, G., Sebald, M. 1980. *Antimicrob. Agents Chemother.* 18:502–5
23. Franke, A. E., Clewell, D. B. 1981. *J. Bacteriol.* 145:494–502
24. Gorbach, S. L., Bartlett, J. G. 1974. *N. Engl. J. Med.* 290:1177–84, 1237–45, 1289–94
25. Gregory, E. M., Dapper, C. H. 1980. *J. Bacteriol.* 144:967–74
26. Gregory, E. M., Dapper, C. H. 1983. *Arch. Biochem. Biophys.* 220:293–300
27. Guiney, D. G., Davis, C. E. 1978. *Nature* 274:181–82
28. Guiney, D. G., Davis, C. E. 1982. *Plasmid* 7:196–98
29. Guiney, D. G., Hasegawa, P., Stalker, D., Davis, C. E. 1983. *J. Infect. Dis.* 147:551–58
30. Hayes, T. J., Cundy, K. R., Fernandes, P. B., Hoober, J. K. 1983. *J. Bacteriol.* 155:1171–77
31. Hirai, N., Kingston, D. G., Van Tassel, R. L., Wilkins, T. D. 1982. *J. Amer. Chem. Soc.* 104:6149–50
32. Holdeman, L. V., Cato, E. P., Moore, W. E. C. 1977. *Va. Polytech. Anaerobe Lab. Manual*, pp. 30–43. Blacksburg, VA. 4th Ed.
33. Holdeman, L. V., Good, I. J., Moore, W. E. C. 1976. *Appl. Environ. Microbiol.* 31:359–75
34. Holland, J. W., Stauffer, L. R., Altemeir, W. A. 1979. *J. Clin. Microbiol.* 10:121–27
35. Hylemon, P. B., Phibbs, P. V. 1974. *Biochem. Biophys. Res. Commun.* 60: 88–95
36. Hylemon, P. B., Young, J. L., Roadcap, R. F., Phibbs, P. V. 1977. *Appl. Environ. Microbiol.* 34:488–94
37. Johnson, J. L. 1978. *Int. J. Syst. Bacteriol.* 28:245–56
38. Johnson, J. L. 1980. *Appl. Environ. Microbiol.* 39:407–13
39. Kasper, D. L. 1976. *J. Infect. Dis.* 133:79–87
40. Kasper, D. L. 1976. *J. Infect. Dis.* 134:59–66
41. Kasper, D. L., Onderdonk, A. B., Crabb, J., Bartlett, J. G. 1979. *J. Infect. Dis.* 140:724–31
42. Kasper, D. L., Onderdonk, A. B., Polk, B. F., Bartlett, J. G. 1979. *Rev. Infect. Dis.* 1:278–88
43. Kasper, D. L., Onderdonk, A. B., Reinap, B. G., Lindberg, A. A. 1980. *J. Infect. Dis.* 142:750–56
44. Kasper, D. L., Seiler, M. W. 1975. *J. Infect. Dis.* 132:440–50
45. Kasper, D. L., Weintraub, A., Lindberg, A. A., Lonngren, J. 1983. *J. Bacteriol.* 153:991–97
46. Kinouchi, T., Ohnishi, Y. 1983. *Appl. Environ. Microbiol.* 46:596–604
47. Kotarski, S. F., Salyers, A. A. 1981. *J. Bacteriol.* 146:853–60

47a. Kotarski, S. F., Salyers, A. A. 1984. *J. Bacteriol.* 158:102–9
48. Kuritza, A. P., Salyers, A. A. 1983. *J. Bacteriol.* 153:1180–86
49. Labbe, M., Delamore, N., Pepersack, F., Crokaert, F., Yourasowsky, E. 1980. *J. Clin. Pathol.* 33:1189–92
50. Leive, L. 1965. *Biochem. Biophys. Res. Comm.* 21:290–96
51. Lindner, J. G., Marceles, J. H., DeVos, N. M., Hoogkamp-Korstanje, J. A. A. 1979. *J. Gen. Microbiol.* 111:93–99
52. Linn, S. P., Chan, T., Lipeski, L., Salyers, A. A. 1983. *J. Bacteriol.* 156:859–66
53. Macrina, F. L., Mays, T. D., Smith, C. J., Welch, R. A. 1981. *J. Antimicrob. Chemother.* 8:77–86 (Suppl. D)
54. Macy, J., Probst, I. 1979. *Ann. Rev. Microbiol.* 33:561–94
55. Magot, M., Fayolle, F., Privitera, G., Sebald, M. 1981. *Mol. Gen. Genet.* 181:559–61
56. Malamy, M. H., Tally, F. P. 1981. *J. Antimicrob. Chemother.* 8:59–75 (Suppl. D)
57. Mancini, C., Behme, R. J. 1977. *J. Infect. Dis.* 136:597–600
58. March, P. K., Malamy, M. H., Shimell, M. J., Tally, F. P. 1983. *Antimicrob. Agents Chemother.* 23:726–30
59. Mayberry, W. R. 1980. *J. Bacteriol.* 144:200–4
60. Mays, T. D., Johnson, J. L. 1979. *Plasmid* 2:299
61. Mays, T. D., Smith, C. J., Welch, R. A., Delfini, C., Macrina, F. L. 1982. *Antimicrob. Agents Chemother.* 21:110–18
62. Miyagawa, E., Azuma, R., Suto, T., Yano, I. 1979. *J. Biochem.* 86:311–20
63. Moore, W. E. C., Holdeman, L. V. 1974. *Appl. Microbiol.* 27:961–79
64. Mossie, K. G., Jones, D. T., Robb, F. T., Woods, D. R. 1980. *Antimicrob. Agents Chemother.* 17:838–41
65. Olsson, B., Dornbusch, K., Nord, C. E. 1979. *Antimicrob. Agents Chemother.* 15:263–68
66. Olsson-Liljequist, B., Dornbusch, K., Nord, C. E. 1980. *Antimicrob. Agents Chemother.* 18:220–25
67. Onderdonk, A. B., Kasper, D. L., Cisneros, R. L., Bartlett, J. G. 1977. *J. Infect. Dis.* 136:82–89
68. Onderdonk, A. B., Kasper, D. L., Mansheim, B. J., Louie, T. J., Corbach, S. L., Bartlett, J. G. 1979. *Rev. Infect. Dis.* 1:291–301
69. Polk, B. F., Kasper, D. L. 1977. *Ann. Intern. Med.* 86:569–71
70. Poxton, I. R. 1979. *J. Clin. Pathol.* 32:294–98
71. Poxton, I. R., Brown, R. 1979. *J. Gen. Microbiol.* 112:211–17
72. Poxton, I. R., Brown, R., Collee, J. G. 1982. *J. Med. Microbiol.* 15:223–31
73. Poxton, I. R., Ip, M. K.-Y. 1981. *J. Gen. Microbiol.* 126:103–10
74. Privalle, C. T., Gregory, E. M., 1979. *J. Bacteriol.* 138:139–45
75. Privitera, G., Dublanchet, A., Sebald, M. 1979. *J. Infect. Dis.* 139:97–101
76. Privitera, G., Sebald, M., Fayolle, F. 1979. *Nature* 278:657–59
77. Rashtchian, A., Booth, S. J. 1981. *J. Bacteriol.* 146:121–27
78. Rashtchian, A., Dubes, G. R., Booth, S. J. 1982. *J. Bacteriol.* 150:141–47
79. Rashtchian, A., Dubes, G. R., Booth, S. J. 1982. *Antimicrob. Agents Chemother.* 22:701–3
80. Riepe, S. P., Galdstein, J., Alpers, D. H. 1980. *J. Clin. Invest.* 66:314–22
81. Roberton, A. M., Stanley, R. A. 1982. *Appl. Environ. Microbiol.* 43:325–30
82. Rotimi, V. O., Duerdin, B. I., Hafiz, S. 1981. *J. Med. Microbiol.* 14:359–70
83. Rudek, W., Haque, R.-V. 1976. *J. Clin. Microbiol.* 4:458–60
84. Salyers, A. A., Arthur, R., Kuritza, A. 1981. *J. Agric. Fd. Chem.* 29:475–80
85. Salyers, A. A., Balascio, J. R., Palmer, J. 1982. *J. Food Biochem.* 6:39–55
86. Salyers, A. A., Harris, C. J., Wilkins, T. D. 1978. *Can. J. Microbiol.* 24:336–38
87. Salyers, A. A., Kotarski, S. F. 1980. *J. Bacteriol.* 143:780–88
88. Salyers, A. A., Lynn, S., Gardner, J. F. 1983. *J. Bacteriol.* 154:287–93
89. Salyers, A. A., O'Brien, M. 1980. *J. Bacteriol.* 143:772–80
90. Salyers, A. A., O'Brien, M., Kotarski, S. F. 1982. *J. Bacteriol.* 150:1008–15
91. Salyers, A. A., O'Brien, M., Kotarski, S. F. 1983. *Can. J. Microbiol.* 29:1532–38
92. Salyers, A. A., Palmer, J. K., Wilkins, T. D. 1977. *Appl. Environ. Microbiol.* 33:1118–24
93. Salyers, A. A., Vercellotti, J. R., West, S., Wilkins, T. D. 1977. *Appl. Environ. Microbiol.* 33:319–22
94. Salyers, A. A., West, S., Vercellotti, J. R., Wilkins, T. D. 1977. *Appl. Environ. Microbiol.* 34:529–33
95. Sato, K., Matsuura, Y., Inoue, M., Mitsuhashi, S. 1982. *Antimicrob. Agents Chemother.* 22:579–84
96. Schumann, J. P., Jones, D. T., Woods, D. R. 1982. *J. Bacteriol.* 151:44–47
97. Scudder, P., Uemura, K., Dolby, J., Fukuda, M. N., Feizi, T. 1983. *Biochem. J.* 213:385–94

98. Schnaitman, C. A. 1971. *J. Bacteriol.* 108:553–63
99. Shimell, M. J., Smith, C. J., Tally, F. P., Macrina, F. L., Malamy, M. M. 1982. *J. Bacteriol.* 152:950–53
100. Siegel, L. S., Hylemon, P. B., Phibbs, P. V. 1977. *J. Bacteriol.* 129:87–96
101. Slade, H. J. K., Jones, D. T., Woods, D. R. 1981. *J. Bacteriol.* 147:685–87
102. Slade, H. J. K., Jones, D. T., Woods, D. R. 1982. *FEMS Microbiol. Lett.* 15:257–59
103. Slade, H. J. K., Shumann, J. P., Parker, J. R., Jones, D. T., Woods, D. R. 1983. *J. Bacteriol.* 153:1545–47
103a. Smith, C. J., Macrina, F. L. 1984. *J. Bacteriol.* 158:739–41
104. Smith, R. D., Salyers, A. A. 1981. *J. Bacteriol.* 145:8–13
105. Smith, C. J., Welch, R. A., Macrina, F. L. 1982. *J. Bacteriol.* 151:281–87
106. Shoemaker, N., Smith, M. D., Guild, W. J. 1980. *Plasmid* 3:80–87
107. Stiffler, P. W., Keller, R., Traub, N. 1974. *J. Infect. Dis.* 130:544–48
108. Stoffel, W., Dittmar, K., Wilmes, R. 1975. *Hoppe-Selyer's Z. Physiol. Chem.* 356:715–25
109. Tajima, M., Sawa, K., Watanabe, K., Ueno, K. 1983. *J. Antibiotics* 36:423–28
110. Tally, F. P., Cucheral, C. J., Jacobus, N. V., Gorbach, S. L., Aldridge, K. E., et al. 1983. *Antimicrob. Agents Chemother.* 23:536–40
111. Tally, F. P., O'Keefe, J. P., Sullivan, N. M., Gorbach, S. L. 1979. *Antimicrob. Agents Chemother.* 16:565–71
112. Tally, F. P., Shimell, M. J., Carson, G. R., Malamy, M. H. 1981. In *Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids*, ed. S. B. Levy, R. C. Clowes, E. L. Koenig, pp. 51–54. New York: Plenum
113. Tally, F. P., Snydman, D. R., Gorbach, S. L., Malamy, M. H. 1979. *J. Infect. Dis.* 139:83–88
114. Tally, F. P., Snydman, D. F. R., Shimell, M. J., Malamy, M. H. 1982. *J. Bacteriol.* 151:686–91
115. Van Soest, P. J. 1978. *Amer. J. Clin. Nutr.* 31:S12–S20 (Suppl.)
116. Van Tassell, R. L., MacDonald, D. K., Wilkins, T. D. 1982. *Infec. Immun.* 37:975–80
117. Varel, V. H., Bryant, M. P. 1974. *Appl. Microbiol.* 28:251–57
118. Vercellotti, J. R., Salyers, A. A., Bullard, W. S., Wilkins, T. D. 1977. *Can. J. Biochem.* 55:1190–96
119. Von Nicolai, H., Hammann, R., Werner, H., Zilliken, F. 1983. *FEMS Microbiol. Lett.* 17:217–20
120. Wallace, B. L., Bradley, J. E., Rogolsky, M. 1981. *J. Clin. Microbiol.* 14:383–88
121. Welch, R. A., Jones, K. R., Macrina, F. L. 1979. *Plasmid* 2:261–68
122. Welch, R. A., Macrina, F. L. 1981. *J. Bacteriol.* 145:867–72
123. Wilkins, T. D., Wagner, D. L., Veltri, B. J., Gregory, E. M. 1978. *J. Clin. Microbiol.* 8:553–57
124. Wollenweber, H. W., Rietschel, E. T., Hofstad, T., Weintraub, A., Lindberg, A. A. 1980. *J. Bacteriol.* 144:898–903
125. Young, F. E., Mayer, L. 1979. *Rev. Infect. Dis.* 1:55–63

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THE DISEASE SPECTRUM, EPIDEMIOLOGY, AND ETIOLOGY OF TOXIC-SHOCK SYNDROME

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INTRODUCTION

In 1978, Todd et al (87) introduced the term toxic-shock syndrome (TSS) to describe an apparently new multi-system disease characterized by the rapid onset of fever, hypotension, erythroderma, and delayed desquamation of the palms and soles. For epidemiologic studies, during the first part of 1980, these clinical criteria were used to establish the case definition (16, 27, 81).

A diagnosis of confirmed TSS is made if all six criteria outlined in Table 1 are met. If one criterion is missing, the diagnosis is that of probable TSS. If a patient has had recurrent episodes of a milder form of the disease, a diagnosis of TSS may be made based on the criteria for recurrent TSS established by the Centers for Disease Control (CDC) (81) and by the TSS Tri-State Epidemiological Study as outlined in Table 2 (27a).

The association of TSS with *Staphylococcus aureus (S. aureus)* made initially by Todd et al (87) has since been confirmed (27, 81). A protein associated with more than 90% of TSS-associated *S. aureus* strains was isolated by Bergdoll et al (10) and initially labeled staphylococcal enterotoxin F (SEF). The emetic action, however, was not reproducible, and the name was changed to toxic shock toxin (TST) (65). Simultaneously, Schlievert et al (73) reported the purification of a biologically active compound called pyrogenic exotoxin C (PEC). Testing of this preparation with antibody specific for TST (SEF) in the Bergdoll laboratory indicated that PEC contained about 20–25% TST (SEF) (unpublished data).

In addition, the amino acid composition of PEC did not correspond to that

Table 1 Toxic-shock syndrome case definition*

1. Fever (temperature ≥ 38.9°C (102°F)
2. Rash (diffuse macular erythroderma)
3. Desquamation, 1–2 weeks after onset of illness, particularly of palms and soles
4. Hypotension (systolic blood pressure ≤ 90 mm Hg for adults or < 5th percentile by age for children < 16 years of age, or orthostatic syncope)
5. Involvement of three or more of the following organ systems:
   - A. Gastrointestinal (vomiting or diarrhea at onset of illness)
   - B. Muscular (severe myalgia or creatine phosphokinase level ≥ 2 × ULN[a])
   - C. Mucous membrane (vaginal, oropharyngeal, or conjunctival hyperemia)
   - D. Renal (BUN[b] or Cr[c] ≥ 2 × ULN or ≥ 5 white blood cells per high power field—in the absence of a urinary tract infection)
   - E. Hepatic (total bilirubin, SGOT[d] or SGPT[e] ≥ 2 × ULN).
   - F. Hematologic (platelets ≤ 100,000/mm^3)
   - G. Central nervous system (disorientation or alterations in consciousness without focal neurologic signs when fever and hypotension are absent)
6. Negative results of the following tests, if obtained:
   - A. Blood, throat, or cerebrospinal fluid cultures
   - B. Serologic tests for Rocky Mountain spotted fever, leptospirosis, or measles

[a] Twice upper limits of normal for laboratory
[b] Blood urea nitrogen level
[c] Creatinine level
[d] Serum glutamic oxaloacetic transaminase level
[e] Serum glutamic pyruvic transaminase level
*These criteria indicate the original case definition. They were modified in the summer of 1981 so that orthostatic dizziness is accepted as evidence of hypotension, and patients with *S. aureus* bacteremia are included as cases if they otherwise meet the case definition. A case is "confirmed" if it meets all the criteria, and "probable" if it is missing only one criterion. (Desquamation is unnecessary in fatal cases.) (16, 27, 58)

found for TST (SEF). Other proteins that react with specific TST (SEF) antibody have now been reported. These different preparations will be referred to as TST, with the individual investigator's name for the protein in brackets.

Although children, men, and nonmenstruating women may develop TSS, the vast majority of recent cases have occurred during menstruation in previously healthy women who have been using tampons (27, 43, 58, 72, 81). The incidence of reported disease has decreased following the publicity associated with the 1980 "outbreak" (Figure 1) (18). Many questions regarding the pathogenesis and specifically the precise role of tampons in this disease remain unanswered.

HISTORY

Illnesses resembling TSS and associated with *S. aureus* have been reported since 1927 (3, 31, 33, 49, 68, 84, 92), and, in fact, TSS may have been the cause of the infamous Bundaberg disaster in Australia in 1928 (66). Shortly

Table 2 Definition of confirmed and probable recurrent episodes of toxic-shock syndrome in women enrolled in the Tri-State Toxic-Shock Syndrome Study (27a)

Confirmed recurrence:

1. Temperature of ≥ 38.9°C (102°F)
2. Rash—diffuse erythroderma
3. Hypotension
   - A. Systolic blood pressure of ≤ 90 mm Hg for an adult (defined as ≥ 16 years) or < the 5th percentile for age for a child; **or**
   - B. Orthostatic hypotension as shown by a decrease in diastolic blood pressure of ≥ 15 mm Hg from a recumbent to a sitting position; **or**
   - C. History of orthostatic dizziness
4. Mucous membrane (vaginal, oropharyngeal, or conjunctival hyperemia or myalgia)
5. Gastrointestinal—history of vomiting or diarrhea at the onset of illness
6. Desquamation (usually fingers, toes, palms, or soles)

Probable recurrence:

Two of the first five criteria for a confirmed recurrence, plus desquamation, plus association with the menstrual period; **or**

Three of the first five criteria for a confirmed recurrence, but no desquamation, plus association with the menstrual period

after Todd et al (87) made their initial observations in 1978, a number of adults now recognized to have had TSS were described as having Kawasaki's disease (32, 52, 78). Between July, 1979 and January, 1980, seven patients with TSS were hospitalized in Madison, Wisconsin. During detailed questioning, it was noted that six of the seven patients had been menstruating at the time of onset of their illness (27). Two of the patients had had similar but milder illnesses with previous menstrual periods, suggesting that the syndrome could recur.

In Minnesota, between December, 1979 and January, 1980, five women with a similar illness were hospitalized, and it was subsequently determined that they also had onset of illness during menstruation (54). These cases and the apparent association of illness with menses was reported to the CDC in January, 1980, by the state epidemiologists in Wisconsin and Minnesota (81). In May, 1980, the CDC reported their findings of the first 55 nationally reported cases (15). Fifty-two (95%) of the cases were in women. Of 40 patients for whom a menstrual history was obtained, 38 (95%) had onset with menses. Thirteen patients had recurrent episodes.

By June, 1980 case control studies statistically linking the onset of menstrual TSS with tampons had been reported by the Wisconsin Division of Health (27) and by the CDC (81). Similar trends were also noted at that time by the Utah Department of Health (43). In September, 1980 the CDC reported that although TSS had been associated with many tampon brands, women using Rely® (The Procter and Gamble Co.) brand tampons were at a greater relative risk of

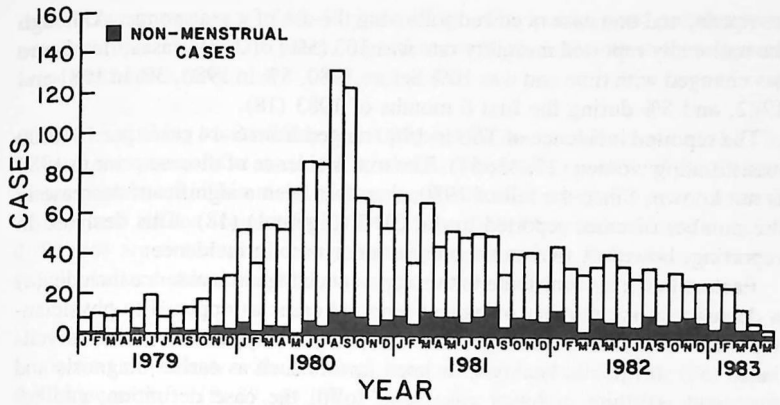


Figure 1 Confirmed cases of toxic-shock syndrome, United States, January 1979 to May 1983. (18)

developing TSS (16, 72). Rely® was immediately and voluntarily withdrawn from the market by the company.

During the time these epidemiologic studies were being conducted, microbiologic studies established that the majority of patients with menstrual TSS had vaginal or cervical colonization with *S. aureus* (27, 81), and that these strains made characteristic marker proteins or toxins SEF & PEC (10, 73). Recent studies have been directed toward establishment of an animal model, characterization of the *S. aureus* gene responsible for the production of the TST marker protein, further characterization of TST, and a determination of factors related to pathogenesis.

EPIDEMIOLOGY

Surveillance and Incidence

Statewide surveillance for cases of confirmed TSS began in Wisconsin and Minnesota in January of 1980 (27, 57), in Utah shortly thereafter (43), and in other states following the national CDC communications on TSS. In 1982, the CDC listed TSS as a nationally reportable disease (17).

Through June 15, 1983 2,204 cases of TSS meeting the case definition (Table 1) had been reported to the CDC (Figure 1) (18). Of these, 2,108 (96%) were in females and 96 (4%) were in males. For 2,203 women for whom the menstrual history was known, 1,824 (90%) had disease onset associated with menstruation. Of the menstruation-associated cases for whom information related to catamenial product use was available, 1,517 (99%) of 1,535 were tampon users; 17 (1%) cases occurred with the exclusive use of napkins or

minipads, and one case occurred following the use of a sea sponge. Although the nationally reported mortality rate was 103 (5%) of 2,107 cases, this figure has changed with time and was 10% before 1980, 5% in 1980, 3% in 1981 and 1982, and 5% during the first 6 months of 1983 (18).

The reported incidence of TSS in 1980 ranged from 6–14 cases per 100,000 menstruating women (27, 43, 57). The true incidence of disease prior to 1980 is not known. Since the fall of 1980, there has been a significant decrease in the number of cases reported to the CDC (Figure 1) (18). This decrease in reporting, however, may not represent a decrease in incidence.

Factors that may contribute to this apparent change in incidence include: (*a*) a decrease in the number of patient-reported cases as opposed to physician-reported cases (26); (*b*) changes in the intensity and type of statewide surveillance (57); (*c*) public health–associated factors such as earlier diagnosis and treatment resulting in fewer cases that fulfill the case definition; and (*d*) tampon-associated factors such as changes in the number of women using tampons, their wearing habits, tampon composition, withdrawal of Rely® brand tampons from the market, and changes in publicity and public recognition of TSS (26).

Although cases have been reported from all fifty states and the District of Columbia, significant differences in incidence have been noted between states. Through June 15, 1983 five states—Wisconsin, Minnesota, Colorado, Utah, and California—accounted for 44% of the total reported cases, although they represent only 16% of the United States population (18). Although intensified surveillance may be a factor, regional differences in the degree of immunity to TST (J. M. Vergeront, personal communication) or in the distribution of toxin producing organisms may be important factors.

Although surveillance for TSS is not generally conducted in most other countries, over 150 cases of confirmed TSS have been reported from these countries (39). When information is available, the degree of association with menses, tampons, and the production of TST by *S. aureus* strains is comparable to that noted in the United States (39). In most countries, tampons account for less than 30% of catamenial product usage as compared to 70% in the United States.

B: Risk Factors in Menstrual TSS:

Since 1980, the vast majority of confirmed cases of TSS have been recognized to occur in previously healthy, young white menstruating women who were using tampons at the time of onset of illness. The explanation for this combination of risk factors is complex.

AGE The mean age of patients with confirmed menstrual TSS is 22.6 years (range 11–61) and this figure has not varied since 1980 (18, 64). One third of

cases occur in teenagers of 15–19 years of age. The mean age of patients with nonmenstrual TSS is 27 years, significantly higher than that for menses-associated cases (64).

RACE A striking racial difference is present for menstrually associated TSS: 97% of such cases have occurred in whites, who make up 83% of the United States population. This racial difference is not as marked for nonmenstrual TSS, where whites account for 87% of cases (64). The reasons for this racial difference are not clear and only partially explained by differences in menstrually related practices (40).

MENSTRUATION AND TAMPON USE The initial observation that a high proportion of patients with TSS had onset during menses (27) has now been well documented (18, 37, 43, 58, 72, 81). The fact that use of tampons is a significant risk factor for the development of TSS has been established by six case-control studies (27, 37, 43, 58, 72, 81). Although these studies varied in a design and methodologic technique, all demonstrated that at least 97% of cases, compared to 76%–89% of matched controls, wore tampons during their period associated with disease onset. Following a review of these studies, it was concluded that the use of tampons was an adequate explanation for the excess risk of TSS in women over men and that discontinuance of the use of tampons would be expected to reduce the incidence of TSS by 80–90% (83).

Although TSS has occurred and continues to occur with use of all brands of tampons, use of Rely® brand tampons and tampons of increased absorbency have been associated with a greater relative risk of developing menstrual TSS. The second CDC case-control study determined that there was a greater relative risk of developing TSS with use of Rely® as compared to other tampon brands, although cases occurred with use of all brands (72). The Tri-State Study determined that tampon absorbency was the best predictor for the risk of acquiring TSS and that the TSS risk associated with the use of Rely® brand tampons was greater than that predicted by absorbency alone (58).

Although continuous use of tampons was associated with a greater risk of TSS than noncontinuous use in one case control study (81), this was not found in a subsequent study (58). No association between TSS and the frequency of changing tampons has been demonstrated.

OTHER FACTORS Factors such as douching, sexual activities, use of other vaginal products, types of clothing, and various personal hygiene practices have been shown not to be significantly associated with TSS (58). In the Tri-State TSS Study, it was found that more cases than controls had a history of vaginitis in the year prior to onset of TSS, more cases than controls had an illness characterized by fever, diarrhea, dizziness, and vomiting during the

menstrual period preceding the period associated with illness, and fewer cases than controls reported leisure-time exercise activities (58). Among those individuals using oral contraceptives, fewer cases (36%) than controls (61%) used oral contraceptives (58).

Nonmenstrual TSS and Associated Risk Factors

As of June 15, 1983, 295 confirmed cases of nonmenstrual TSS had been reported to the CDC (18). Although the mean age is higher and the ratio of males to females closer to that in the general population, the clinical findings and complications are the same as those of the patients with menstrual TSS (63). The number of confirmed cases reported in children under ten years is surprisingly low (62). Nonmenstrual cases have been associated with nonsurgical cutaneous and subcutaneous infections (30%), childbirth or abortion (27%), infections following a wide variety of surgical procedures (18%) including gauze packing of a nasal cavity, vaginal infections occuring at times other than during menses or the postpartum interval or following the use of a diaphragm (5%), and other or unknown sources of infection (20%) (63).

Typically, surgical wounds that are colonized with *S. aureus* and responsible for postoperative TSS have minimal or no signs of inflammation. The incubation period for postoperative or immediate postpartum TSS is of 12–48 hrs (7, 63). Relatively few cases have been associated with deep-tissue infections.

CLINICAL SPECTRUM AND THERAPY

Clinical Presentation

SEVERE DISEASE Multi-system organ damage and massive capillary vasodilatation with intravascular fluid loss comprise the apparent toxin-mediated damage that results in TSS. Prolonged hypotension following the loss of intravascular volume may result in ischemic organ damage that further compounds the toxin-mediated damage. The onset of illness is abrupt, with symptoms and signs of moderate to severe disease including fever, chills, malaise, headache, sore throat, myalgias, muscle tenderness, fatigue, vomiting, diarrhea, abdominal pain, and orthostatic dizziness or syncope (Figure 2).

Over the next 24–48 hours, diffuse erythema, severe watery diarrhea often with incontinence, decreased urine output, cyanosis, and edema of the extremities may be noted. Cerebral ischemia, edema, or a toxin-mediated effect on the central nervous system rapidly results in somnolence, confusion, irritability, agitation, and occasionally hallucinations (19, 34, 37a, 50, 81, 87, 89).

During initial physical examination of a moderately to severely ill patient, fever, tachycardia, tachypnea, a low or unobtainable blood pressure,

erythroderma, and muscle—particularly abdominal muscle—tenderness are noted, in conjunction with peripheral cyanosis and edema, conjunctival hyperemia and hemorrhages, beefy red edematous mucous membranes, somnolence, disorientation, and agitation. In menstrual TSS, edema and erythema of the inner thighs and perineum may be noted along with cervicovaginal erythema, edema, discharge, and ulcerations in conjunction with a normal uterine and adnexal examination. In nonmenstrually associated cases, vaginitis or another focus of infection will generally be present.

The variety of abnormalities in clinical laboratory tests reflects shock and organ failure, but some may be due to a direct action of the toxin. The total number of mature and immature neutrophils usually exceeds 90%, and profound lymphopenia is often noted (19). Thrombocytopenia and anemia are present over the first few days, frequently accompanied by prolonged prothrombin and partial thromboplastin times. Sterile pyuria and elevated BUN and creatinine levels reflect kidney damage, abnormalities in tests of liver function reflect liver damage (19, 36a), and profound hypocalcemia may

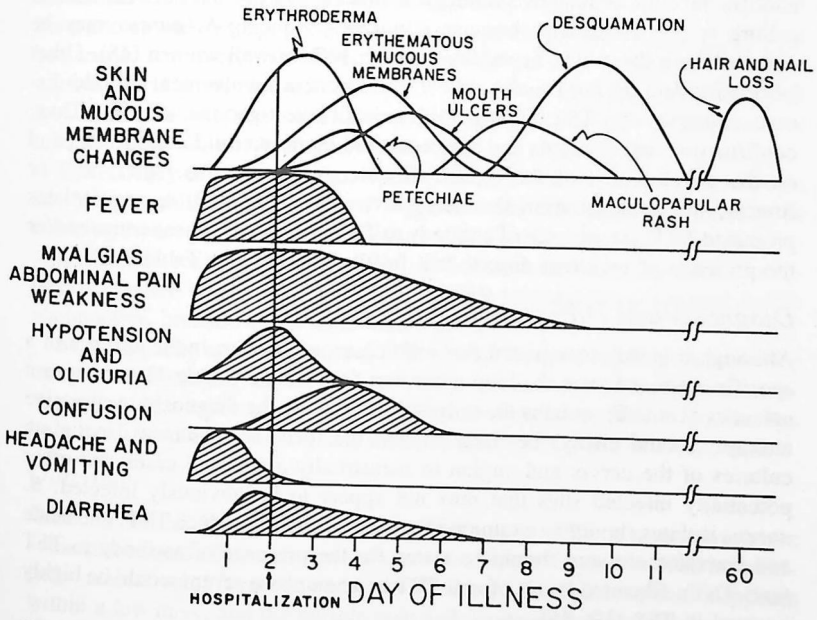


Figure 2 Composite drawing of major systemic, skin, and mucous membrane manifestations of toxic-shock syndrome. (Reproduced with permission from Chesney et al (19).

reflect both hypoproteinemia and high serum levels of a circulating calcitonin-like material (22). Frequently, an elevated CPK is noted, indicating muscle involvement. The hypophosphatemia that occurs despite impaired renal function is unexplained. *S. aureus* may be cultured from the cervix or vagina in more than 85% of patients with menstrual TSS (27, 81). Antibody to TST will be absent at the onset of disease in ≥ 86% of patients (10, 85). The majority of these tests will have returned to normal by seven to ten days after onset of illness.

MILD DISEASE Recognition of mild episodes of TSS is particularly important for menstrual TSS. Such patients typically do not develop antibody to TST during convalescence (10, 85) and may develop one or more recurrences without appropriate therapy (27, 27a). Such recurrences may be mild or severe (27, 27a, 50, 81, 89).

The presence of any combination of fever, headache, rash, sore throat, diarrhea, vomiting orthostatic dizziness, syncope, or myalgias in a menstruating woman should raise the possibility of TSS (20). A specific laboratory test to confirm the clinical diagnosis is not yet available. The presence of *S. aureus* on culture is not diagnostic, because non-TST–producing *S. aureus* may be cultured from the cervix or vagina of up to 10% of well women (48). Other laboratory data do not usually reflect multi-system involvement in mild disease, and assays for TST in body fluids are still investigational (51, 90). Thus, confirmation that the signs and symptoms did represent mild TSS will depend on the development of the typical desquamation of palms, soles, toes or fingers, the demonstration that any cervical or vaginal *S. aureus* isolates produced TST, the absence of antibody to TST in an acute phase serum, and/or the presence of recurrent disease that fulfill the criteria in Table 2.

Diagnosis and Differential Diagnosis

Although it is well recognized that mild cases may occur, in the absence of a specific diagnostic test the case definition for a single (Table 1) or recurrent episodes (Table 2) remains the only way of making the diagnosis. Aggressive attempts should always be made to find the focus of *S. aureus*, including cultures of the cervix and vagina in menstrually associated cases and other potentially infected sites that may not appear to be obviously infected. *S. aureus* isolates should be examined for their ability to produce TST, and acute and convalescent sera should be tested for the presence of antibody to TST (anti-TST). Elevated levels of anti-TST in acute phase serum would be highly unusual in TSS (10, 85).

The differential diagnosis of TSS includes those clinical entities in which the rapid onset of fever, rash, hypotension, and multi-system involvement are

observed. Sepsis, septic shock (particularly meningococcemia), scarlet fever, leptospirosis, Rocky Mountain Spotted fever, fulminant viral syndromes, urinary tract infection, severe toxin-induced diarrhea, and Kawasaki's syndrome may be confused with TSS.

Therapy

The most serious problems encountered in the first 24–48 hours of hospitalization include hypotension, myocardopathy with arrhythmias, pulmonary edema with shock lung or adult respiratory distress syndrome, acidosis, hyponatremia and associated electrolyte disorders, acute renal failure, hypocalcemia, and disseminated intravascular coagulation. The rapid restoration of intravascular volume to achieve adequate tissue perfusion should be accomplished immediately, using fluids and, if necessary, vasopressors. In severe cases, adequate hemodynamic monitoring in an intensive care setting is necessary to allow early detection of cardiac dysfunction, adult respiratory distress syndrome, and bleeding abnormalities (19, 34, 50, 81, 87, 89).

Specific therapy involves eradication of *S. aureus* and removal of TSS-associated toxin(s). Antibiotics do not appear to be critical for recovery from the acute episode except possibly in the rare patient who has a bacteremia or bacteruria. Antimicrobials appear to be most important for eradication of the organism and prevention of recurrences (27, 27a, 28). The effectiveness of local measures such as removal of foreign bodies and drainage and irrigation of infected areas has not been evaluated.

The efficacy of a short course of methylprednisolone or dexamethasone has not been established. It has been suggested that they may be beneficial in reducing the length of fever and severity of disease if given early enough (88).

As the TSS toxin(s) has not yet been definitely established, specific recommendations for its elimination cannot be made because the usual sites for its conjugation, breakdown, or elimination are not known.

Because patients with menstrual TSS typically do not develop antibody for many months and are subject to recurrences, cultures and antibody determinations should be repeated every 2–3 months to determine if the *S. aureus* has been eradicated and a significant antibody titer to TST has developed (85).

Outcome

Once the initial life-threatening complications of TSS have resolved, the subsequent course is usually one of rapid resolution (Figure 2). Characteristically, resolution of fever is noted in 48–72 hours, the erythroderma disappears within a few days, and the muscle pain and weakness resolves in 7–10 days. Oral ulcerations and glossitis with a strawberry tongue may be present initially. Fine facial and truncal desquamation may begin after several days, whereas the

characteristic peripheral finger, toe, palm, and sole full thickness desquamation does not usually occur until 10–21 days after disease onset. A late-onset pruritic, maculopapular rash with edema and fever, probably unrelated to antimicrobials, occurs in more than 50% of patients 7–10 days after disease onset (21).

Death associated with TSS has been reported as late as 15 days after hospitalization and has been attributed most often to refractory cardiac arrhythmias, irreversible respiratory failure, and bleeding due to coagulation defects (46).

Sequelae attributed to TSS that appear to be related to prolonged periods of hypotension include chronic renal failure, gangrene, and loss of hair and nails (telogen effluvium) (21, 87).

Neuromuscular and neurophsychologic sequelae that may be less clearly related to hypotension included prolonged muscle weakness and pain, peripheral neuropathies, and carpal tunnel syndrome (21, 69). Impaired memory and poorly sustained concentration have been described in patients who required only intravenous fluids to restore blood pressure (67). These findings lead to speculation that the TSS-associated toxin(s) may have a direct effect on nerve or muscle tissue.

Recurrences

Recurrent episodes of TSS appear to be unique to menstrually associated cases; recurrences associated with nonmenstrual cases rarely have been reported. Most recurrent episodes occur within the first three months after the initial episode, and the first episode is usually the most severe, although exceptions to both statements have been reported (27, 27a).

The recurrence rate for untreated patients maybe as high as 65% (28). The rate of recurrences has been shown to decrease significantly if tampon use is discontinued and a course of antistaphylococcal antibiotics is given (27, 27a). In Wisconsin, the recurrence rate has dropped dramatically in association with the use of antibiotics and discontinuation of tampon use. The recurrence rate was 65% (17/26) prior to 1980, 20% (15/74) in 1980, and 0% (0/29) after 1980 (27a, 85).

Serologic studies have determined that 88% of well males and females over 20 years of age have a titer of greater than 1:100 of antibody to TST (SEF) as determined by RIA (91). In contrast, less than 15% of patients with TSS have an acute titer of greater than 1:5 and only 45% of women with menstrual TSS develop a titer of greater than 1:100 one year after their initial illness (85). This failure to make antibody to a protein closely associated with the organism may provide an indicator of continuing susceptibility to the disease.

PATHOLOGY AND PATHOGENESIS

Histopathologic Findings

The histopathologic findings at autopsy support the concept that TSS is a toxin-mediated disease. Typically, there is a total absence of tissue invasion by bacteria and minimal evidence of an inflammatory reaction in most organs. Findings felt to be due to direct effects of the toxin and unrelated to hypoperfusion have been: (*a*) ulcerations in the cervix, vagina, esophagus, and bladder, (*b*) lymphocyte depletion in the lymph nodes, (*c*) subepidermal cleavage plane in the skin, and (*d*) the mild inflammatory changes present in the kidney, liver, heart, and muscle (46, 59).

Cervico-vaginal ulcerations are the only characteristic lesions noted in the genital tract in fatal cases. These ulcerations have been noted on postmortem examination of a patient with menstrual TSS who had never used tampons (46). These ulcerations are superficial, with capillary vasodilatation and thrombosis with inflammation of the mucosa present, but without deep tissue bacterial invasion. The layer of vacuolization and separation in the ulcers occurs beneath the basal layer. The same type of ulcer has also been found in the bladder and esophagus, suggesting that these ulcerations may be due to the toxin and not to tampons (46).

Although the myocardium was described as normal in one series of fatal cases of TSS (59), in another series of eight autopsies all patients had evidence of focal round cell infiltration with variable degrees of congestion, edema, and hemorrhage (46). Myxoid degeneration was found in all heart valves available from four patients in one series (46). Sections of muscle have demonstrated only congestion, edema, focal hemorrhage or fiber necrosis, and a mild acute inflammatory infiltrate (46, 59).

Varying degrees of triaditis or periportal lymphocytic inflammation have been the most consistent finding in the liver, although a centrilobular congestion with necrosis and mild cellular degeneration has also been described (46, 59). In the kidney, toxin-mediated mononuclear interstitial nephritis may result from an initial perivasculitis of the adventitia of the renal venules (59), lesions that probably precede the hypotension-induced acute tubular necrosis. The most characteristic findings in the spleen and lymph nodes have been lymphocyte depletion, inactive hypocellular, hypoplastic lymphoid follicles with edema, and marked histiocytosis in the interfollicular areas (46, 59).

Perivascular lymphocytic infiltrates and bullae that separate at the basement membrane are characteristic of the early skin changes in TSS (46, 59). There has been no evidence of a vasculitis.

The immediate cause of death in TSS is not always clear. In one group of nine patients, seven died of a refractory cardio-ventricular arrhythmia, one of

an acute respiratory arrest, and one as a result of a coagulation defect (46). Noncardiogenic pulmonary edema was the only clinical development closely associated with a fatal outcome (46).

Pathogenesis

The most intriguing issue relating to the pathogenesis of TSS is that of determining how the combined events of TST-producing *S. aureus*, TST antibody–negative individuals, age, race, menses, and tampons have merged so facilely over the last few years to produce a dramatic increase in the incidence of this disease. TST-producing strains of *S. aureus* were present in the 1950s (1). The prevalence of antibody to TST (SEF) in a general population was the same in 1960 and 1980 (91). Thus, the recent increase in incidence of TSS must be the result of other newly introduced cofactors.

Several theories relating to the role of tampons in inducing bacterial growth or stimulating toxin formation have been proposed. Tampons do not appear to enhance the growth of TST (PEC)–producing *S. aureus* isolates in either nutrient broth or human blood (14, 47). Tampons of different types, however, can alter the amount of TST (SEF) produced in vitro by as much as 300-fold (47) when compared to non–tampon containing controls. The in vivo significance of this finding has not yet been established.

A second proposed explanation for the role of tampons in TSS is that they may remove substrates that normally act to inhibit the growth of *S. aureus* (70). The anaerobic lactobacilli present in the vagina in high concentrations during menses may inhibit the growth of *S. aureus* as they do the growth of *Neisseria gonorrhoeae*. In some in vitro experiments the inhibitory effect of vaginal lactobacilli is enhanced by the presence of other substrates. It has been suggested that tampons may absorb these substrates, thus eliminating or reducing their inhibitory effect (70) and permitting the growth of *S. aureus*.

A third theory relating to the role of tampons suggests that cellulases produced by certain aerobic gram-negative bacteria that may be part of the vaginal flora breakdown carboxymethyl-cellulose in tampons into reducing sugars (86). These sugars would enrich the vaginal microenvironment, encouraging the growth of *S. aureus*. However, high glucose concentrations have been found to inhibit TST (PEC) production (77).

A fourth theory relating tampons to TSS pathogenesis deals with the role of tampons in creating cervico-vaginal ulcerations that enhance toxin(s) absorption. The superficial microulcerations produced following the brief insertion of tampons in healthy women (35) and the chronic ulcers produced by the long term continuous use of tampons (5, 41) are very different histologically from the ulcerations seen in TSS even in a non–tampon user (46). In addition, the intact vaginal mucosa is a very absorptive surface, and there is probably no need to invoke mucosal ulcerations to explain toxin absorption.

A fifth theory relating to the possible role of tampons suggests that the tampon may act to optimize conditions of pH and oxygen concentration for toxin production (93).

Unrelated to tampons, neutrophil (PMN) dysfunction has been invoked as a possible mechanism for the pathogenesis of TSS. A transient decrease in the bactericidal activity of blood PMN's during menses has been demonstrated (12). No difference has been noted, however, in the bactericidal capacity of PMN's against TSS and control strains of *S. aureus* grown in the presence of Rely® tampons in another study (8).

Preferential adherence of TSS *S. aureus* isolates to vaginal epithelial cells from TSS patients does not appear to be a pathogenetic factor. Adherence to cells obtained from five healthy women and five women recently recovered from TSS was the same for TSS *S. aureus* isolates (8).

The role of endotoxin in the pathogenesis of TSS is unclear (36). The enhanced susceptibility of rabbits to endotoxin following the administration of TST (PEC) suggests that endotoxin may be important (74).

THE ORGANISM

Todd et al (87) initially associated *S. aureus* with TSS. The *S. aureus* isolates from five patients with this disease were lysed by group I bacteriophages, but further characterization of the organisms was not reported. Other investigators have since reported that about 60% of the *S. aureus* strains isolated from TSS patients were lysed by group I phages, with phage 29 being the most frequently encountered (1, 4, 27, 30, 81). The remainder of the strains were lysed by phages from the other phage groups or were nontypable. A number of previously nontypable strains can now be typed using the new phages developed by Altemeier et al (2). Most of the TSS *S. aureus* strains are penicillin- (30, 44), arsenate- (4, 30, 44), and cadmium-resistant (30, 44). In general, these strains seem to be less hemolytic than non-TSS strains (4, 23, 86) although this appears to be more in degree of hemolytic activity rather than being totally nonhemolytic (23).

Characterization of TST and TST-Like Proteins

The *S. aureus* strains initially studied by Todd et al (87) produced an exotoxin that caused a positive Nikolsky sign in the newborn mouse but was distinct from the exfoliatin responsible for the staphylococcal scalded-skin syndrome. Although 70% of TSS *S. aureus* strains produce this epidermal toxin, the correlation is insufficient to attribute the illness to this toxin except as a contributing factor when it is produced (42).

Bergdoll et al (10) initially reported the isolation of a new marker protein produced by more than 90% of TSS strains examined. This protein was labeled enterotoxin F (SEF) because it produced emesis in monkeys. When the emetic

action of the protein was not reproducible, even when 30 $\mu g\ kg^{-1}$ was injected intravenously (65), the name was changed to toxic-shock toxin (TST). At about the same time, Schlievert et al (73) reported the identification of a similar new protein that they called pyrogenic exotoxin C (PEC). Using the TST-specific antibody, about 20–25% of the PEC preparation appears to be identical to TST (Bergdoll, unpublished data). Others have now reported purification of a similar protein identified as TST by use of the TST-specific antibody (4, 24, 51, 56, 60). These proteins have been called SEF (56), TSS marker protein (51), and toxic shock marker protein (TSMP) (60).

TST is a simple protein of molecular weight 24,000 daltons. It has an isoelectric point around pH 7.0 and is resistant to tryptic digestion but degradable by pepsin at pH 4.5 (65). Its amino acid composition is similar to that of the staphylococcal enterotoxins, including the two half-cystine residues (Table 3). The molecular weight of the Schlievert preparation (PEC) is 22,000 and the pI is 7.2 (73). PEC has a different amino acid composition than that reported by Bergdoll et al (Table 3).

The fact that 75–80% of PEC is different than TST using anti-TST antibody may account for the differences in the amino acid composition. These differences in the proportion of TST in the different preparations make comparisons

Table 3 Amino acid composition of toxic-shock toxin purified by Reiser et al (65) and Schlievert et al (73)

Amino acid	TST[a]	TST (PEC)[b]
	Number of residues[c]	
Lysine	23	14
Histidine	5	15
Arginine	4	4
Aspartic acid	27	34
Threonine	22	10
Serine	23	12
Glutamic acid	18	47
Proline	13	0
Glycine	15	30
Alanine	4	25
Half-Cystine	2	0
Valine	6	9
Methionine	2	3
Isoleucine	16	6
Leucine	17	10
Tyrosine	10	2
Phenylalanine	7	3
Tryptophane	2	—

[a] Calculated from data of Reiser et al (65)
[b] Calculated from data of Schlievert et al (73) using molecular weight of 24,000
[c] Calculated using a molecular weight of 24,000

of relative biologic activity difficult. The preparation of TST purified by Notermans & Dufrenne (56) had a molecular weight of 23,000 daltons and a pI of 7.2. Following polyacrylamide gel electrophoresis and testing with anti-TST antibody, only a small amount of non-TST material was found to be present.

Association of TST with *S. aureus* strains has been reported by a number of investigators. Through the use of anti-TST antibody, Bergdoll et al (11) reported that 130 of 142 TSS strains produced TST, with an additional seven strains producing one or more of the other staphylococcal enterotoxins (11). More recently, 203 of 235 TSS strains (86.4%) were found to produce TST; an additional 16 strains produced one or more of the other enterotoxins but not TST (Bergdoll, unpublished data). Schlievert and colleagues (73, 75) found in two separate studies of 28 and 135 TSS strains that all tested strains produced PEC. Analysis of 90 strains of *S. aureus* by each of the two laboratories (Bergdoll & Schlievert) showed a difference in results for 13 strains. Four strains that were PEC positive using the isoelectric focusing method were negative using the TST-specific antibody. Nine strains that were positive using the TST-specific antibody were negative by the PEC-focusing method (1). Others who have used the TST-specific antibody in their testing of TSS *S. aureus* strains have found 80–82% of isolates to produce TST (13, 29, 30).

Two additional proteins have been identified using sodium dodecyl sulfate polyacrylamide electrophoresis. These proteins were associated with 25 of 32 TSS isolates examined (24). Both proteins reacted with antisera taken from a TSS patient and later were shown to react with the specific antibody to TST (Cohen, personal communication). These proteins were isolated from cell extracts and had molecular weights of 30,000 and 33,000 daltons. It is possible that they represent precursors of the TST and as such may have higher biological activity.

Nakashima & Wuepper have identified a protein called TSS-1 that is produced by TSS strains grown in RMPI 1640 medium in dialysis casings implanted into the peritoneal cavities of rabbits (55). This protein has a molecular weight of 25,000 daltons, a pI of 7.2, and nonspecific mitogenic activity against human and rabbit lymphocytes but not against mouse, rat, and guinea pig lymphocytes. This protein is not identical to TST; TST activity was not neutralized by the antibody to TSS-1, nor did TST give a precipitin reaction with this antibody in gels.

TSS *S. aureus* strains have been reported to produce a substance that is a potent inducer of human leukocytic pyrogen and lymphocyte-activating factor (38). This substance was identified with a 20,000- to 25,000-dalton fraction obtained by gel filtration of crude supernatant fluids from TSS strains. The specific compound in this fraction responsible for the activity was not identified, but it is probably TST because others (60) have reported that TST (TSMP) was also able to induce production of human interleukin-1.

Many *S. aureus* strains produce one or more of the biologically active staphylococcal enterotoxins. Enterotoxin B and some of the other enterotoxins have been shown to produce in monkeys the same signs and symptoms observed in TSS (9). A number of TSS *S. aureus* strains produce one or more of the enterotoxins in conjunction with the TST, while several others produce only one of the enterotoxins, primarily enterotoxin B (Bergdoll, unpublished data). The significance of this finding is not yet clear.

Production of TST

Schlievert & Blomster (77) have studied the effects of different in vitro laboratory conditions on the production of TST (PEC). They found that more toxin was produced at 37°C and 40°C than at 30°C, even though growth of the organism was similar at 30°C and 37°C. Little or no toxin was produced under anaerobic conditions. Glucose concentrations of greater than 0.3% in the media (beef heart supplemented with NaCl, glutamine, and either acetate or bicarbonate phosphate) inhibited the production of TST (PEC). The effect of pH on the production of the toxin is difficult to interpret because the pH during the incubation decreased from the starting pH to a pH of 6.0–6.2. The largest quantities of toxin were produced at starting pHs of 7.0 and 8.0; no organism growth or toxin production occurred at starting pHs of 5.0 and 9.0.

The acid production in the medium during incubation in this study (77) is in contrast to the production of ammonia in the pancreatic digest of casein medium used by others (Bergdoll, unpublished data). In the latter case, the pH rises during incubation and maximum toxin production occurs at starting pHs of 4.5 and 5.0 with final pH being 7.6–7.8. No toxin or growth was obtained at a starting pH of 4.0, and no toxin was produced at a starting pH of 8.0 even though good growth was obtained (final pH 8.3) (Bergdoll, unpublished data). It would appear that the optimum pH for the toxin production is around 7.0, as in both media the pH for toxin production is near 7.0 and the pH at some time during incubation is 7.0. Further work is needed to clarify factors that alter the in vitro production of TST.

Tampons can significantly alter the production of TST in vitro (47), although they do not alter the growth of the organism (14, 47). The amount of toxin produced in vitro can vary from undetectable to 300 $\mu g\ ml^{-1}$ depending on the particular brand and style of tampon. Under the conditions of these experiments, toxin production did not vary at pH values as low as 4.5, although the growth of the organism was retarded.

Studies of the genetics of TST production failed to demonstrate plasmid or phage involvement or linkage with known chromosomal genes (75). One report suggested that most TSS *S. aureus* strains harbor prophages with common plating characteristics and suggested that the toxins involved in TSS may be transmitted by lysogenic conversion (79). However, others demonstrated that

the gene controlling TST production was not transferred by lysogeny (45). The latter group cloned the gene in *Escherichia coli* and showed that the cloned product was serologically and biologically identical to the TST produced by the parent *S. aureus* strain (45). The TST determinant is associated with a larger DNA segment, which is absent or rearranged in TST-negative strains. The relationship of the prophage to transmittal of TST production by lysogenic conversion became questionable when it was shown that one of the indicator strains used in the lysogeny studies (79) produced TST (45).

Biological Properties of TST

IMMUNE FUNCTION TST has been shown to alter lymphocyte function in several in vitro systems. TST (PEC) is a nonspecific T-cell mitogen (73). Mitogenicity was evaluated using rabbit splenocytes and human peripheral T and B cell populations. A minimal response of the B cells to TST (PEC) was demonstrated, whereas it was an active mitogen for the T-cell–enriched population. Using the slide modification of the Jerne plaque assay, TST (PEC) has been shown to suppress IgM antibody synthesis of both murine and rabbit spleen cells to sheep erythrocytes (73). TST (PEC) plus endotoxin further reduced the plaque-forming cell response compared to TST (PEC) alone (76).

In vivo, although rabbits can make antibodies to TST (10, 73), the response does not occur in all animals. Of six animals receiving a total of six subcutaneous injections of TST (50 μg of PEC each) over 105 days, only three developed antibody to TST (PEC). All animals were capable of making antibodies to sheep erythrocytes, however, suggesting the presence of a specific unresponsiveness to TST (PEC) rather than immunodeficiency (73). Neither TST (PEC) nor endotoxin given alone suppressed the immune response of rabbits to sheep erythrocytes; however, when TST (PEC) was given intravenously and followed four hours later by endotoxin, the immune response was significantly suppressed when measured after four days. In animals receiving a higher dose of endotoxin, the immune response remained suppressed for up to twelve days (76).

Suppression of the clearance of colloidal carbon by the reticulo-endothelial system (RES) was found to be unaffected by intravenous injection of TST (PEC). However, when TST (PEC) was given and followed by intravenous endotoxin four hours later, significant RES blockage occurred (76).

An additional biological activity of TST (PEC) is that of skin sensitivity (76). Rabbits given a subcutaneous injection of TST (PEC) every other week developed progressive increases in skin test responsiveness to homologous toxin. Reaction diameters increased with progressive increase in the size of the challenge doses of TST (PEC). In addition, rabbits previously sensitized only

to purified protein derivative (PPD) of *Mycobacterium tuberculosis* demonstrated an enhanced skin-test reactivity when challenged with PPD plus TST (PEC) as compared to challenge with PPD or TST (PEC) alone.

NONIMMUNE BIOLOGICAL FUNCTION In vivo, TST (PEC and TSSMP) have been demonstrated to be pyrogenic for rabbits (51, 73). Antibody to TST (PEC) prevents the pyrogenic response (73).

Both TST (PEC) and TST (TSSMP) have been demonstrated to be lethal for rabbits (51, 76). The susceptibility of rabbits to TST (PEC) appeared to depend on prior conditioning of the animals. Animals quarantined for one week prior to use, given tetracycline for visible signs of pulmonary infection, and restrained for three hours on a pyrogen test rack were relatively resistant to TST (PEC). Animals that were not conditioned were highly susceptible to TST (PEC) (76). Approximately 50% of these animals were killed by a single injection of toxin and 100% died after three daily doses. Melish et al (51) established 60 μg of TST (TSSMP) kg^{-1} as the LD_{50} for rabbits.

The lethality of TST (PEC) for rabbits is markedly enhanced following the administration of small doses of endotoxin (74), a phenomenon known as enhanced endotoxin susceptibility. In experiments demonstrating this phenomenon, *S. typhimurium* endotoxin with an LD_{50} of 500 μg kg^{-1} was used (74). If rabbits were given an intravenous dose of 100 μg kg^{-1} of TST (PEC), followed by endotoxin at four hours, the LD_{50} dose of endtoxin was 0.01 μg kg^{-1}, a 50,000-fold enhanced susceptibility to endotoxin. Using a range of doses, a straight line was obtained when the log of the TST (PEC) pretreatment dose was plotted against the log LD_{50} of endotoxin.

Death occurred in rabbits 1–24 hours after challenge, depending on the dose used, and after an illness characterized by fever, hypothermia, diarrhea, and labored respiration. Other characteristics of this phenomenon included loss of the enhanced endotoxin susceptibility 21–24 hours after the endotoxin administration and no enhanced susceptibility using low doses of TST (PEC) unless two hours elapsed before repeated endotoxin administration.

In the rabbit model, when methyl-prednisolone or pooled human gammaglobulin were given prior to the administration of TST (PEC) or endotoxin, mortality was significantly decreased. Although no single agent was protective after the administration of endotoxin, the combined administration of methyl-prednisolone and gammaglobulin decreased mortality. Ibuprofen was ineffective under all conditions (61).

The most important animal model thus far examined is the baboon. Quimby (personal communication) found that the intravenous administration of TST (SEF) into adult female baboons was associated with a marked decrease in blood pressure (60/45 mm Hg), orthostatic hypotension, ascites, hyperthermia, rash (abdomen and thighs), conjunctival hyperemia, and thrombocytopenia, as well as chemical evidence for abnormalities involving muscle, liver, and kidney.

Log phase cultures of 11 of 16 TSS-associated *S. aureus* strains grown in beef-heart dialysate were found to be lethal in rabbits when injected into subcutaneous polyethylene chambers (80). No deaths occurred in rabbits injected with control strains. Fever, diarrhea, and labored breathing occurred in most cases, and all deaths occurred within 48 hours. Two of the TSS strains that produced relative large amounts of TST (pI 7.2) were not lethal in the rabbits, and another TST-producing strain was lethal in only one of four rabbits. Thus, it may be that TST did not account for the lethal action of these strains but rather another biologically active substance produced by the *S. aureus*.

Although it has not been established that TST is the cause of TSS, this toxin has been found in the sera, urine, and vaginal washings of TSS patients (51) as well as in the breast milk of a woman with TSS (90).

PREVALENCE OF COLONIZATION BY TSS-ASSOCIATED *S. AUREUS:*

Cervico-Vaginal Flora

Depending on the study, *S. aureus* has been isolated from cervical or vaginal cultures of between 0–17% of well nonmenstruating women. Whereas in most studies the colonization rate was less than 5% (6, 25, 71), higher figures of 17% were obtained from positive cervical cultures from 9 of 52 women during menses (17%) (82) and 14 of 81 postpartum women (17%) (48). Thus, cervico-vaginal *S. aureus* colonization appears to be highest during menses and the postpartum period.

The prevalence of TST-producing *S. aureus* isolates was recently examined prospectively in 600 women being seen for routine pregnancy-related or problem-associated visits (48). Of these 600 individuals, 55 (9%) had a positive labial or vaginal culture for *S. aureus*. Seven women (1%) were colonized with TST (SEF)–producing isolates.

Other Flora

Anterior nares cultures from 11 of 22 healthy children were positive for *S. aureus*, and 4 of these strains produced TST (PEC) (74). In families of women with vaginal strains of TST-producing *S. aureus*, 3 of 18 family members were colonized with TST (SEF)–producing *S. aureus* (48).

The finding that more than 60% of people over 10 years of age and 88% of individuals over age 20 years have antibody to TST suggests that the prevalence of colonization with TST-producing *S. aureus* strains in childhood may be high or that intermittent colonization with these strains may be frequent.

SUMMARY

From this composite picture of the history and recent developments related to TSS, several points are clear. TSS is not a new disease, and TST-producing strains of *S. aureus* are not new. What is new is the recent dramatic increase of this disease in young women who use tampons during menses and who lack antibody to TST. What is also new is the recognition that the disease commonly recurs but only in menstrually associated cases. What remains to be determined are the precise role of tampons, the factors leading to toxin induction, and the mechanism of action of this potent toxin. In order to better determine what these factors and mechanism of action are, and to determine if the TST marker protein is in fact the critical toxin, a reliable animal model is badly needed. Finally, a reliable laboratory test to confirm the clinical diagnosis is another high-priority need. The further unraveling of the secrets of this complex disease may greatly enhance our understanding of the disease associated with this toxin, of the intricacies of toxin production by other bacteria, and of the role that exogenous cofactors play in disease processes.

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Literature Cited

1. Altemeier, W. A., Lewis, S. A., Schlievert, P. M., Bergdoll, M. S., Bjornson, H. S., et al. 1982. *Ann. Intern. Med.* 96:978–82
2. Altemeier, W. A., Lewis, S. A., Bjornson, H. S., Staneck, J. L., Schlievert, P. M. 1983. *Arch. Surg.* 118:281–84
3. Aranow, H. Jr., Wood, W. B. Jr. 1942. *J. Am. Med. Assoc.* 119:1491–95
4. Barbour, A. G. 1981. *Infect. Immun.* 33:442–49
5. Barrett, K. F., Bledsoe, S., Greer, B. E., Droegemueller, W. 1977. *Am. J. Obstet. Gynecol.* 144:386–88
6. Bartlett, J. G., Onderdonk, A. B., Drude, E., Goldstein, C., Anderka, M., et al. 1977. *J. Infect. Dis.* 136:271–77
7. Bartlett, P., Reingold, A. L., Graham, D. R., Dan, B. B., Selinger, D. S., et al. 1982. *J. Am. Med. Assoc.* 247:1448–50
8. Bassaris, H. P., Venezio, F. R., Morlock, B. A., Phair, J. P. 1981. *J. Infect. Dis.* 144:386–88
9. Beisel, W. R. 1972. *Toxicon* 10:433–40
10. Bergdoll, M. S., Crass, B. A., Reiser, R. F., Robbins, R. N., Davis, J. P. 1981. *Lancet* 1:1017–21
11. Bergdoll, M. S., Crass, B. A., Reiser, R. F., Robbins, R. N., Lee, A. C. M., et al. 1982. *Ann. Intern. Med.* 96:969–71
12. Berger, E. M., Harada, R. H., Bowman, C. M., Murphy, K., Repine, J. E. 1983. *Clin. Res.* 31(1):48A
13. Bonventure, P. F., Weckbach, L., Staneck, J., Schlievert, P. M., Thompson, M. 1983. *Infect. Immun.* 40:1023–29
14. Broome, C. V., Hayes, P. S., Ajello, G. W., Feeley, J. C., Gibson, R. J., et al. 1982. *Ann. Intern. Med.* 96:959–62
15. Centers for Disease Control. 1980. *Morbid. Mortal. Weekly Report* 29:229–30
16. Centers for Disease Control. 1980. *Morbid. Mortal. Weekly Report* 29:441–45
17. Centers for Disease Control. 1982. *Morbid. Mortal. Weekly Report* 32:18

18. Centers for Disease Control. 1983. *Morbid. Mortal. Weekly Report* 32:398–400
19. Chesney, P. J., Davis, J. P., Purdy, W. K., Wand, P. J., Chesney, R. W. 1981. *J. Am. Med. Assoc.* 246:741–48
20. Chesney, P. J., Slama, S. L., Hawkins, R. L., Wand, P. J., Crass, B. A., et al. 1981. *New Engl. J. Med.* 304:1426
21. Chesney, P. J., Crass, B. A., Polyak, M. B., Wand, P. J., Warner, T. F., et al. 1982. *Ann. Intern. Med.* 96:847–51
22. Chesney, R. W., McCarron, D. M., Haddad, J. G., Hawker, C. D., DiBella, F. P., et al. 1983. *J. Lab. Clin. Med.* 101:576–85
23. Chow, A. W., Gribble, M. J., Bartlett, K. H. 1983. *J. Clin. Microbiol.* 17:524–28
24. Cohen, M. L., Falkow, S. 1981. *Science.* 211:842–44
25. Corbishley, C. M. 1977. *J. Clin. Pathol.* 30:745–48
26. Davis, J. P., Vergeront, J. M. 1982. *J. Infect. Dis.* 145:449–57
27. Davis, J. P., Chesney, P. J., Wand, P. J. 1980. *N. Engl. J. Med.* 303:1429–35
27a. Davis, J. P., Osterholm, M. T., Helms, C. M., Vergeront, J. M., Wintermeyer, L. A., et al. 1982. *J. Infect. Dis.* 145:441–48
28. Davis, J. P., Chesney, P. J., Vergeront, J. M. 1984. *Medical Microbiology*, Vol. 4. London: Academic In press
29. De Nooij, M. P., Van Leeuwan, W. J., Notermans, S. 1982. *J. Hyg.* 89:499–505
30. De Saxe, M., Wienke, A. A., De Azevedo, J., Arbuthnott, J. P. 1982. *Ann. Intern. Med.* 96:991–96
31. Dunnet, W. B., Schallibaum, E. M. 1960. *Lancet* 2:1227–29
32. Everett, E. D. 1979. *J. Am. Med. Assoc.* 242:542–43
33. Feldman, C. A. 1962. *N. Engl. J. Med.* 267:877–78
34. Fisher, R. F., Goodpasture, H. C., Peterie, J. D., Voth, D. W. 1981. *Ann. Intern. Med.* 94:156–63
35. Friedrich, E. G., Siegesmund, K. A. 1980. *Obstet. Gynecol.* 55:149–56
36. Fumarola, D. 1979. *Lancet* i:221
36a. Gourley, G. R., Chesney, P. J., Davis, J. P., Odell, G. B. 1981. *Gastroenterology* 81:928–31
37. Helgerson, S. D., Foster, L. R. 1982. *Ann. Intern. Med.* 96:909–11
37a. Helms, C. M., Lengeling, R. W., Pinsky, R. L., Myers, M. G., Koontz, F. P., et al. 1981. *Am. J. Med. Sci.* 282:50–60
38. Ikejima, T., Dinarello, C. A., Gill, M. D., Wolff, S. M. 1983. *Clin. Res.* 31:496A
39. Institute of Medicine, National Academy of Science. 1982. National Academy Press, Washington
40. Irwin, C. E. Jr., Millstein, S. G. 1982. *Am. J. Pub. Health.* 72:464–67
41. Jimerson, S. D., Becker, J. D. 1980. *Obstet. Gynecol.* 56:97–99
42. Kapral, F. A. 1982. *Ann. Intern. Med.* 96:972–74
43. Kehrberg, M. W., Latham, R. H., Haslam, B. T., Hightower, A., Tanner, M., et al. 1981. *Am. J. Epidemiol.* 114:873–79
44. Kreiswirth, B. N., Novick, R. P., Schlievert, P. M., Bergdoll, M. S. 1982. *Ann. Intern. Med.* 96:974–77
45. Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., et al. 1983. *Nature* 305:709–912
46. Larkin, S. M., Williams, D. N., Osterholm, M. T., Tofte, R. W., Posalaky, Z. 1982. *Ann. Intern. Med.* 96: 858–64
47. Lee, A. C., Crass, B. A. 1983. *Am. Soc. for Microbiol. Abstr.* B27:59
48. Linneman, C. C., Staneck, J. L., Horstein, S., Barden, T. P., Rauh, J. L., et al. 1982. *Ann. Intern. Med.* 96:940–44
49. McCloskey, R. W. 1973. *Ann. Intern. Med.* 78:85–87
50. McKenna, U. G., Meadows, J. A. III, Brewer, N. S., Wikon, W. R., Perrault, J. 1980. *Mayo Clin. Proc.* 55:663–72
51. Melish, M. E., Chen, F. S., Murata, S. M. 1982. *Clin. Res.* 31:122A
52. Milgrom, H., Palmer, E. L., Slovin, S. F., Morens, D. M., Freedman, S. D., et al. 1980. *Ann. Intern. Med.* 92:467–70
53. Deleted in proof
54. Minnesota Department of Health. 1980. *Dis. Control Newsletter* Vol. 7(8)
55. Nakashima, H., Wuepper, K. D., 1983. *Clin. Res.* 31:149
56. Notermans, S., Dufrenne, J. B. 1982. *Antonie Van Leeuwenhoek* 48:447–55
57. Osterholm, M. T., Forfang, J. R. 1982. *J. Infect. Dis.* 145:458–64
58. Osterholm, M. T., Davis, J. P., Gibson, R. W., Mandel, J. S., Wintermeyer, L. A., et al. 1982. *J. Infect. Dis.* 145:431–40
59. Paris, A. L., Herwaldt, L. A., Blum, D., Schmid, G. P., Shands, K. N., et al. 1982. *Ann. Intern. Med.* 96:858–64
60. Parsonnet, J., Eardley, D., Pier, G. B. 1983. *Intersci. Conf. Antimicrob. Agents Chemother.* Abstr. 766:225
61. Peterson, P. K., Schlievert, P. M., Conroy, W., Kelly, J. A., Spika, J., et al. 1983. *J. Infect. Dis.* 147(2):358
62. Reingold, A. L., Dan, B. B., Shands, K. N., Broome, C. V. 1982. *Lancet* i:1–4
63. Reingold, A. L., Hargrett, N. T., Dan,

B. B., Shands, K. N., Strickland, B. Y., et al. 1982. *Ann. Intern. Med.* 96:871–74
64. Reingold, A. L., Hargrett, N. T., Shands, K. N., Dan, B. B., Schmid, G. P., et al. 1982. *Ann. Intern. Med.* 96: 875–80
65. Reiser, R. F., Robbins, R. N., Khoe, G. P., Bergdoll, M. S. 1983. *Biochemistry.* 22:3907–12
66. Report of the Royal Commission. 1928. *Med. J. Australia,* July 2–12
67. Rosene, K. A., Copass, M. K., Kastner, L. S., Nolan, C. M., Eschenbach, D. A. 1982. *Ann. Intern. Med.* 96:865–70
68. St. Geme, J. W. Jr., Bush, B. M., George, B. L. 1976. *Pediatrics* 57:148–50
69. Sahs, A. L., Helms, C. M., DuBois, C. 1983. *Arch. Neurol.* 40:414–15
70. Sanders, C. C., Sanders, W. E. Jr., Fagnant, J. E. 1982. *Am. J. Obstet. Gynecol.* 142:977–82
71. Sautter, R. L., Brown, W. J. 1980. *J. Clin. Microbiol.* 11:479–84
72. Schlech, W. F., Shands, K. N., Reingold, A. L., Dan, B. B., Schmid, J. P., et al. 1982. *J. Am. Med. Assoc.* 248:835–39
73. Schlievert, P. M., Shands, K. N., Dan, B. B., Schmid, G. P., Nishimura, R. D. 1981. *J. Infect. Dis.* 143:509–16
74. Schlievert, P. M. 1982. *Infect. Immun.* 36:123–28
75. Schlievert, P. M., Kelly, J. A. 1982. *Ann. Intern. Med.* 96:982–86
76. Schlievert, P. M. 1983. *J. Infect. Dis.* 147:319–98
77. Schlievert, P. M., Blomster, D. A. 1983. *J. Infect. Dis.* 147:236–42
78. Schlossberg, D., Kandra, J., Kreiser, J. 1979. *Arch. Dermatol.* 115:1435–36
79. Schutzer, S. E., Fischetti, V. A., Zabriskie, J. B. 1983. *Infect. Immun.* 39:383–87
80. Scott, D. F., Kling, J. M., Kirkland, J. J., Best, G. K. 1983. *Infect. Immun.* 39:383–87
81. Shands, K. N., Schmid, G. P., Dan, B. B., Blum, D., Guidotti, R. J., et al. 1980. *N. Engl. J. Med.* 303:1436–42
82. Smith, C. B., Noble, V., Bensch, R., Ahlin, P. A., Jacobson, J. A. et al., 1982. *Ann. Intern. Med.* 96:948–51
83. Stallones, R. A. 1982. *Ann. Intern. Med.* 96:917–20
84. Stevens, F. A. 1927. *J. Am. Med. Assoc.* 88:1957–58
85. Stolz, S. J., Davis, J. P., Vergeront, J. M., Crass, B. A., Bergdoll, M. S., et al. 1982. *Intersci. Conf. Antimicrob. Agents Chemother.* Abstr. 371:131
86. Tierno, P. M., Hana, B. A., Davies, M. B. 1983. *Lancet* i:615–18
87. Todd, J., Fishaut, M., Kapral, F., Welch, T. 1978. *Lancet* ii:1116–18
88. Todd, J. K., Ressman, M., Caston, S. A., Wiesenthal, A. M. 1982. *Intersci. Conf. Antimicrob. Agents Chemother.* Abstr. 368:131
89. Tofte, R. W., Williams, D. N. 1981. *Ann. Intern. Med.* 94:149–56
90. Vergeront, J. M., Evenson, M. L., Crass, B. A., Davis, J. P., Bergdoll, M. S., et al. 1983. *J. Infect. Dis.* 148:692–98
91. Vergeront, J. M., Stolz, S. J., Crass, B. A., Nelson, D. B., Davis, J. P., et al. 1983. *J. Infect. Dis.* 148:692–98
92. Vic-Dupont, M. P., Duval, E. T., Kamali, J. 1965. *Soc. Med. Hop. Paris* 116:51–59
93. Wagner, G., Bohr, L., Wagner, P., Petersen, L. N. 1984. *Am. J. Obstet. Gynecol.* 148(2):147–50

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THE STRUCTURE AND MODE OF ACTION OF GLYCOPEPTIDE ANTIBIOTICS OF THE VANCOMYCIN GROUP

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INTRODUCTION

The vancomycin group of antibiotics is a family of glycopeptide antibiotics, all members of which possess closely related chemical structures. Vancomycin, which lends its name to the group as a whole, was the first to be discovered, in 1956 (28). Interest in vancomycin and other members of the group has continued to this day. All members of this group were obtained from various species of actinomycetes, typically isolated from soil samples of widely

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varying origins, and all are active against gram-positive bacteria. A summary of the sources and uses of these antibiotics is given in Table 1.

Vancomycin was introduced into clinical use soon after its discovery but was soon superceded by the ß-lactam antibiotics, which were less toxic. Vancomycin continued to be used only for severe staphylococcal infections in cases where, for various reasons, other antibiotics could not be employed. Recently it has become apparent that much of the toxicity in earlier preparations of vancomycin was due to impurities. This problem has now been largely overcome (9). There is now much interest in studying further the properties of vancomycin in clinical use, often in synergistic combination with other antibiotics (26). This is because not only is there well-known resistance to ß-lactam antibiotics, but there has recently been increased observation of infections due to gram-positive organisms. This has also prompted interest in the new antibiotic teichomycin A_2 (8), which is known to be more active than vancomycin (34).

Ristocetin, although bactericidal like vancomycin, had to be withdrawn from use as an antibiotic because it causes aggregation of blood platelets. However, this property was put to good use in helping to diagnose von Willebrand's disease (19). Some patients with this disease lack a plasma protein that is required for ristocetin-induced aggregation. Many further details relating to the clinical uses of vancomycin and ristocetin may be found in a recent review dealing with the vancomycin group, in which the structure and mode of action are also discussed (36). An account has recently been given of the clinical uses of vancomycin (12). Uses of antibiotic A-4696 are discussed in the relevant patent literature (7) and those of avoparcin in various sources (1).

Table 1 Producing organisms and uses of the vancomycin-group antibiotics

Antibiotic	Producing organism	Ref.	Use	Ref.
Vancomycin	*Streptomyces orientalis*	(28)	Clinical: against severe infections	(12)
Ristocetin (identical with ristomycin)	*Nocardia lurida* *Proactinomyces fructiferi*	(14) (2)	Clinical: diagnosis of von Willebrand's disease; study of blood platelet aggregation	(19)
Avoparcin (LL-AV290)	*Streptomyces candidus*	(23)	animal feed additive	(1)
Actinoidin	*Nocardia actinoides*	(41)	Investigation of ristocetin-induced blood platelet aggregation	(36)
A-4696 (actaplanin)	*Actinoplanes missouriensis*	(7)	animal feed additive	(7)
A35512	*Streptomyces candidus*	(29)	animal feed additive	(29)
Teichomycin A_2	*Actinoplanes teichomyceticus*	(34)	investigational	(34)

STRUCTURE OF THE ANTIBIOTICS

The structure elucidation of vancomycin and ristocetin took many years of careful work by several groups, and a few details have only just been determined. In the case of the other antibiotics, research could be modeled on the earlier studies, so progress was faster. Work continues on some of these less well-known members of the group. The body of literature on the structural determinations is large and not necessarily of interest to the microbiologist. Much of it is summarized in another recent review (46), though some corrections and new work have appeared since. We shall, therefore, in general present the results rather than the details of the many experiments involved; additionally, some recently obtained results are discussed. Techniques used have included numerous classical chemical methods, mass spectrometry, high-field proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, and, in the case of vancomycin, X-ray crystallography.

All the members of the group may be described by the formula given in Figure 1; all the substituents, if the structural details have been published, are summarized in Table 2. Figure 1 clearly shows that the antibiotics are all linear heptapeptides. The unusual amino acid residues are linked by several phenolic oxidative couplings. All members of the group except vancomycin have seven aromatic rings; ironically, the first-discovered member of the group is the odd one out, having only five. All the compounds share identical oxygen substitution patterns on rings four, five, six, and seven, whereas R_2 and R_3 are invariably either hydrogen or chlorine atoms. It should be noted that the identity of X_3 in vancomycin, which makes the amino acid residue at this position asparagine, has only just been determined (17).

This discovery was delayed because the X-ray structure for vancomycin was actually obtained from a crystalline product, CDP-I (40), which was obtained by heating vancomycin under weakly acidic conditions (pH 4.2). It is now thought that this caused an isomerization of asparagine to *iso*-aspartic acid to occur. Recent ^{1}H NMR evidence showed that this isomerization had also affected the three-dimensional structure. Thus Figure 2 shows the structure of vancomycin with stereochemical detail. It is found that the chlorine atom on ring two is at the "back" (48), unlike that in CDP-I (40), which is at the "front" (additionally, an amido group in vancomycin is hydrolysed to a carboxyl group in CDP-I).

The stereochemical details of some other members of the group have been elucidated using mainly ^{1}H NMR spectroscopy. The structure of ristocetin is shown in Figure 3, where it can be seen to be very like that of vancomycin, given the differences in X_3 and X_1. This relatively invariant three-dimensional structure is the key to the molecular basis of the antibiotics' action, as will be shown below. As in the case of vancomycin, only recently have certain structural features been elucidated unequivocally. Thus the chiral center of the

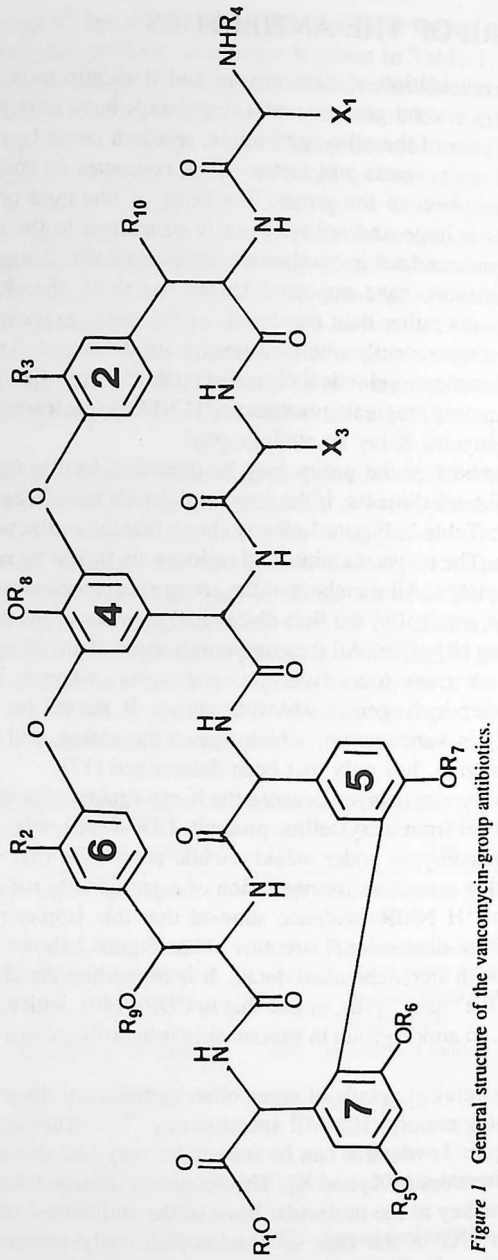
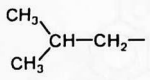
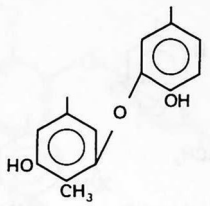
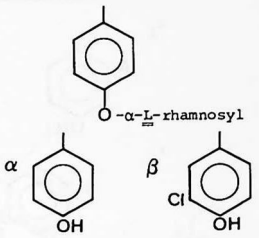
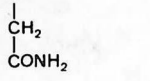


Figure 1 General structure of the vancomycin-group antibiotics.

Table 2 Substituents of the vancomycin group antibiotics (see Figure 1)

Substituent	vancomycin	ristocetin	avoparcin
X_1			
X_3			
R_1	H	CH_3	H
R_2	Cl	H	Cl
R_3	Cl	H	H
R_4	CH_3	H	CH_3
R_5	H	H	H
R_6	H	O-α-D-mannopyranosyl	H
R_7	H	H	H
R_8	O-α-L-vancosaminyl-(1→2)-O-β-D-glucopyranosyl	A ristotetrose[a] B ristobiose[b]	O-α-L-ristosaminyl-(1→2)-O-β-D-glucopyranosyl
R_9	H	O-α-L-ristosaminyl	O-α-L-ristosaminyl
R_{10}	OH	OH	O-α-D-mannopyranosyl

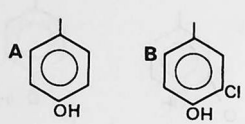
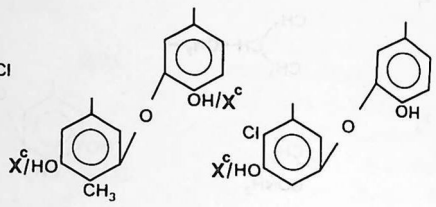
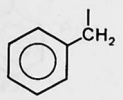
[a] O-α-D-arabinofuranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-O-β-D-glucopyranosyl

[b] O-α-L-rhamnopyranosyl-(1→6)-O-β-D-glucopyranosyl

N-terminus, (4-hydroxyphenyl)glycine, is now thought to have the *R* absolute configuration (18), whereas previously, chemical studies had indicated this configuration for ristomycin (25) (now known to be identical with ristocetin) but ^{1}H NMR had indicated the *S* configuration for ristocetin (45). The chemical evidence is more reliable in this case; indeed, very recent ^{1}H NMR studies are in agreement (47). The stereochemistry of antibiotic A35512B has just been elucidated, using techniques similar to those used for vancomycin and ristocetin (20).

It is noteworthy that in many cases, the antibiotics are not produced as single compounds. Thus ristocetin exists as A and B forms that differ only in the sugars attached to ring four (R_8). Actinoidin also has forms A and B, differing only by one chlorine atom, whereas avoparcin has similarly differentiated compounds α and ß. Antibiotic A35512 is a complex of six factors, of which B

Table 2 continued

Substituent	actinoidin	A-4696	A35512B[a]
X_1			
X_3			
R_1	H	CH_3	CH_3
R_2	H/Cl	Cl	H
R_3	Cl/H	H	H
R_4	H	H	H
R_5	O-D-mannopyranosyl	glucosyl,	H
R_6	H,H[b]	mannosyl,	fucosyl/mannosyl[b]
R_7		rhamnosyl, H[b,d]	H
R_8	O-L-acosaminyl-(1→2)-O-D-glucopyranosyl		rhamnosylglucosyl?[b]
R_9	O-L-actinosaminyl	O-L-ristosaminyl	3-epi-L-vancosaminyl
R_{10}	OH	H	OH

a major factor in complex, see text.
b i.e. position of sugar(s) not fully determined, see c.
c X represents possibility that sugar is attached at this phenolic position.
d sugars present in varying amounts in the different factors, see text.

is the main one (29). Actaplanin (A-4696) appears to be an even more complex mixture (7). In the last case, the difference between most of the factors appears to consist of the presence of varying amounts of the sugars glucose, mannose, and rhamnose.

The presence of very similar aglycones (non–sugar containing portions) but very variable types and amounts of sugars is seen to be a feature of these antibiotics. Just as some of the aromatic amino acid residues are very unusual, so are some of the sugars, such as vancosamine and ristosamine, that were named for the antibiotics that contained them. Although we now have an insight into how the antibiotics work on a molecular level, as described below, the reason for the presence of many features of the structures remains a matter for speculation.

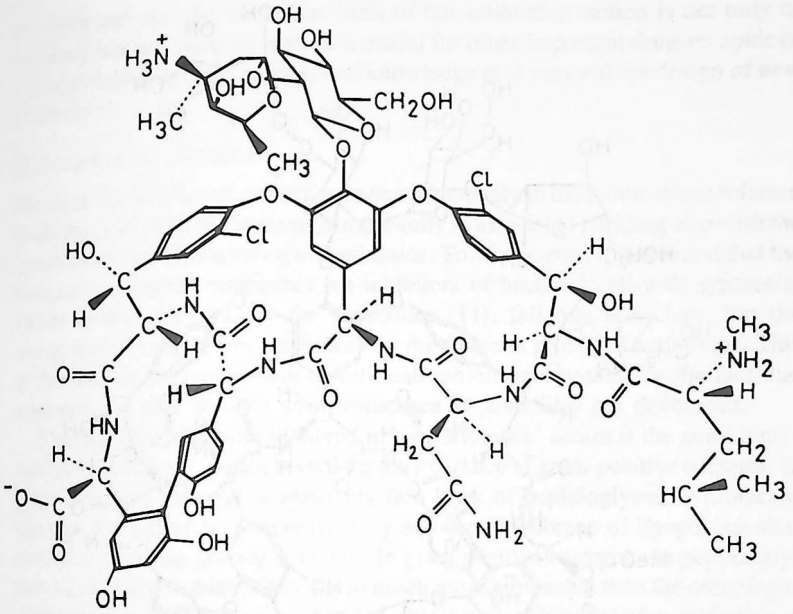


Figure 2 The structure of vancomycin, showing stereochemical detail.

BIOSYNTHESIS: VANCOMYCIN AND RISTOCETIN

Some details of the biosynthesis of vancomycin and ristocetin have been elucidated. In the case of vancomycin, a culture of *Streptomyces orientalis* was supplemented with either [1,2-$^{13}C_2$]acetate, (2*RS*)-[2-^{13}C]-(4-hydroxyphenyl)glycine [*p*-HPG], or one of three differently isotopically-substituted tyrosines. This showed, using ^{13}C NMR spectroscopy, that the (3,5-dihydroxyphenyl)glycine (the residue containing ring seven in Figure 1) could be derived from acetate, whereas (4-hydroxyphenyl)glycine and (3-chloro)-ß-hydroxytyrosine may be derived from tyrosine. Therefore, feeding of *p*-HPG led to enrichment of the residues containing rings four and five, whereas feeding of tyrosine led also to enrichment in the residues containing rings two and six (16).

For ristocetin, similar feeding experiments were carried out with *Nocardia lurida*. In this case, there are two additional aromatic residues to account for. It was found that the aromatic ring of X_3 (Figure 1) is also derived from acetate; in other words, both of the (3,5-dihydroxyphenyl)glycine units arise in this way. The other residue, X_1, was shown to be derived from *p*-HPG (15).

In the case of vancomycin, it remains as a point of interest to find out how the chlorine atoms are incorporated; the variable presence of these atoms in the

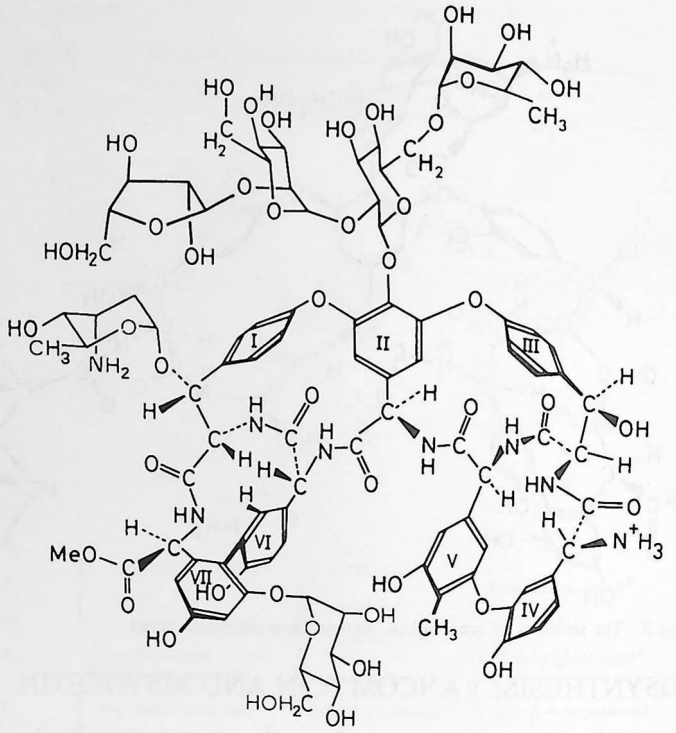


Figure 3 The structure of ristocetin A, showing stereochemical detail.

antibiotics is an interesting feature. For all the antibiotics, the source of the sugars remains to be determined; only the most tentative ideas have been put forward (34).

MODE OF ACTION

It is important to differentiate biochemical studies on the mode of action of vancomycin and other members of the group from studies of the molecular basis of antibiotic action. Thus, practically all the biochemical studies, usually on vancomycin, were carried out when the structures were unknown. These studies nevertheless uncovered much about how the antibiotics work. The structural elucidations alluded to above were, however, a prerequisite for understanding how individual molecules interact with one another.

Both types of study are important; the second could not take place without the first. It is analogous to the use of a magnifying glass to look carefully at a small portion of a picture. The reasons for carrying out the latter, detailed type

of study are that the molecular basis of the antibiotics' action is not only of inherent interest but may also be a model for other important drug-receptor or enzyme-substrate interactions, and knowledge of it may aid the design of new antibiotics.

Biochemical Studies

Much of the biochemical work is reviewed not only in the publications referred to above, (36, 46), but somewhat more fully in a book (11) dealing also with the mode of action of various other antibiotics. To summarize, it was found that the vancomycin-group antibiotics are inhibitors of bacterial cell-wall synthesis. Other antibiotics, notably the penicillins (11), fall into this class, but the mechanism of inhibition is different for the different groups of antibiotics. This difference in the mechanism of cell-wall inhibition accounts for the fact that vancomycin may be used when resistance to penicillins has developed.

The cell-wall structure involved in the antibiotics' action is the peptidoglycan (mucopeptide), which is vital for the existence of gram-positive bacteria. In gram-negative bacteria, a relatively thin layer of peptidoglycan is protected against, for example, vancomycin by an outer membrane of lipopolysaccharide-phospholipid-protein structure. In gram-positive bacteria, the peptidoglycan layer is much thicker but is also much more permeable than the outer layer of the gram-negative species. A full discussion of antibiotics and peptidoglycan metabolism has been published (13), and a complete account of the cell walls of microorganisms is available (38).

Vancomycin is found to be rapidly adsorbed onto the cell walls of gram-positive bacteria to such an extent that much of it cannot be specifically bound to the site of action. A key finding in the elucidation of the mode of action was that vancomycin made complexes with the peptidoglycan precursor UDP-*N*-acetylmuramylpentapeptide (5), and, by stepwise degradations, Perkins was able to show that an acyl-D-alanyl-D-alanine terminus was the feature essential to the complex formation (35). The simplest molecule that will form a complex was found to be *N*-acetyl-D-alanyl-D-alanine (Ac-D-Ala-D-Ala). Importantly, it was found that no significant combination occurred if one or more residues had the L configuration or if the carboxyl group was not free.

These experiments were performed using several techniques, particularly differential ultraviolet spectrophotometry. Although the exact molecular weights of vancomycin and ristocetin were not known when these and subsequent studies (31–33) were carried out, the variation in order of magnitude of the binding constants observed appears to be correct. The data have been widely quoted (11, 36, 38, 46). Although the experiments of Perkins and coworkers usually were carried out using solutions of antibiotics and relativly small peptides, it is likely that the growing points of the peptidoglycan in bacteria will contain similar free D-Ala-D-Ala termini. Therefore, one might

assume that the molecular basis of action found in vitro is analogous to that found in vivo.

The following situation could thus be envisaged. A portion of cell wall of, for example, *Staphylococcus aureus* appears as shown in Figure 4. A transpeptidase enzyme attaches a $(Gly)_5$ residue to the carbonyl carbon of the D-Ala group, which displaces the terminal D-Ala group that was present before cross-linking occurred. In the presence of an antibiotic, the situation shown in Figure 5 is thought to occur. This shows an *N*-acetylmuramylpentapeptide, to which vancomycin or ristocetin are known to bind, with the antibiotic indicated as a hatched area. Thus, possibly, the antibiotics prevent the transpeptidase from completing the cross-linking process and thus stop the cell wall from forming properly.

It is found that vancomycin (31), unlike ristocetin (33), also complexes effectively with *N*-acetyl-L-alanyl-D-glutamyl-glycine, which is analogous to

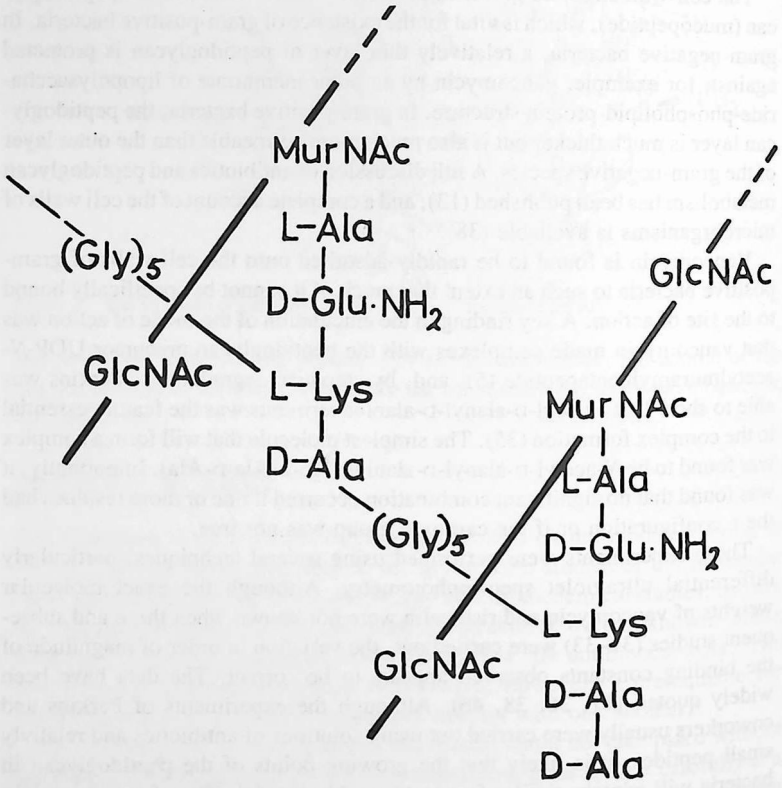


Figure 4 A portion of the cell-wall peptidoglycan of *Staphylococcus aureus*.

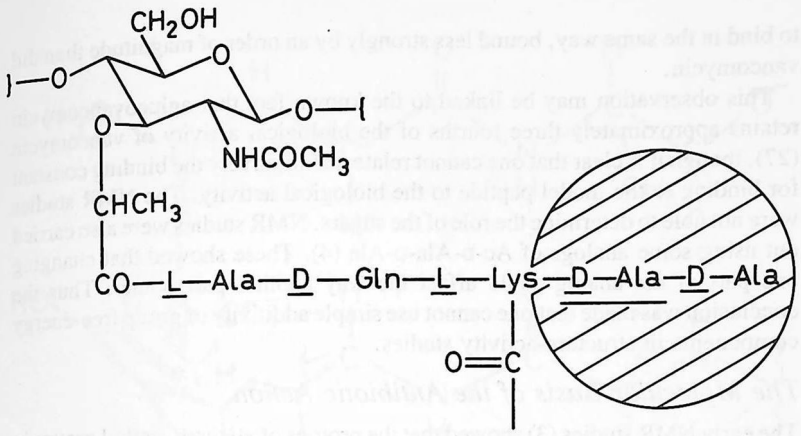


Figure 5 An *N*-acetylmuramylpentapeptide fragment, showing binding to a D-Ala-D-Ala carboxyl terminus by a vancomycin-group antibiotic. Binding indicated by hatched area.

part of the peptide side-chain of various *Micrococcus* organisms. This fits with the observation that vancomycin binds to the cell walls of this type of bacteria much more strongly than does ristocetin (42). Similarly, it has been found that analogs of other cell-wall variants bind to the antibiotics, sometimes better to vancomycin and sometimes to ristocetin. Many of the fine details of these interactions have not been worked out.

It has been pointed out (11) that the exact site of action of the antibiotics is still not certain. Thus it has not been proven that the UDP-*N*-acetylmuramylpentapeptide is the exact compound to which the antibiotics bind in vivo. Nevertheless, although this point remains problematic, more than one group chose to study the interactions of the antibiotics with the small molecules representing the simplest cell-wall analogs.

Nieto & Perkins (32, 33) had been able to deduce, using ultraviolet spectroscopy, circular dichroism, and optical rotatory dispersion, some details of the nature of the binding with Ac-D-Ala-D-Ala and closely related molecules. They could say that ionic interaction, hydrogen bonding, steric repulsion, and hydrophobic bond formation were involved. It was clear that the phenolic groups of vancomycin and ristocetin were close to the binding site because their UV characteristics were altered on binding. However, Perkins could not propose a detailed binding site, because the structure of vancomycin was not known at the time.

The same was true for the first ^1H NMR studies (3), which used the proton chemical shifts of the three methyl groups of Ac-D-Ala-D-Ala as a probe for monitoring the binding with vancomycin. These gave results in broad agreement with those of Perkins and coworkers, but some interesting further information was obtained. Aglucovancomycin, although qualitatively appearing

to bind in the same way, bound less strongly by an order of magnitude than did vancomycin.

This observation may be linked to the known fact that aglucovancomycin retains approximately three fourths of the biological activity of vancomycin (27), though it is clear that one cannot relate quantitatively the binding constant for binding to the model peptide to the biological activity. The NMR studies were not able to determine the role of the sugars. NMR studies were also carried out using some analogs of Ac-D-Ala-D-Ala (4). These showed that changing one part of the analog could affect the way another part bound. Thus the conclusion was made that one cannot use simple additivity of group free-energy components in structure-activity studies.

The Molecular Basis of the Antibiotic Action

The early NMR studies (3) showed that the protons of all three methyl groups in Ac-D-Ala-D-Ala, particularly those of the C-terminal alanine, give signals at higher field (smaller chemical shift) when complexed to vancomycin. This means they are shielded; it could be concluded that they lie over aromatic rings that produce a shielding "ring current." The nature of the binding site could not be more precisely defined until the structure of vancomycin was known.

As in many fields of chemical or biochemical research, a key step was made using X-ray crystallography. As described above, it was possible to obtain an X-ray–derived structure for a crystalline derivative of vancomycin, CDP-I (40). Although there are more structural differences between CDP-I and vancomycin than was thought at the time, most of the stereochemistry of the peptide backbone of CDP-I is the same as that found for vancomycin itself by more recent ^{1}H NMR studies (48).

Using a space-filling molecular model derived from the X-ray structure, it was possible to see how a molecule of Ac-D-Ala-D-Ala could fit into a "cleft" on the front of the molecule, and the type of binding proposed was in accordance with earlier (3, 4) and then recently obtained (44) ^{1}H NMR evidence. The proposed interactions (40) are shown in Figure 6; three hydrogen bonds (dashed lines) between carbonyl groups and N*H* groups were postulated. This binding model has been refined in detail since it was proposed, and additional interactions have been found; nonetheless all of the interactions shown in Figure 6 are retained in current models of the binding.

Following this breakthrough, based on the fortunate ability of CDP-I to form crystals, it was the binding of ristocetin to Ac-D-Ala-D-Ala that was first studied successfully in detail using ^{1}H NMR. This is because free and bound peptide molecules are exchanging sites more slowly in the case of ristocetin than in that of vancomycin. This means that on the time scale of an NMR experiment, separate signals can be seen for the protons of bound and free peptide and antibiotic at room temperature or above for ristocetin. For vancomycin, only

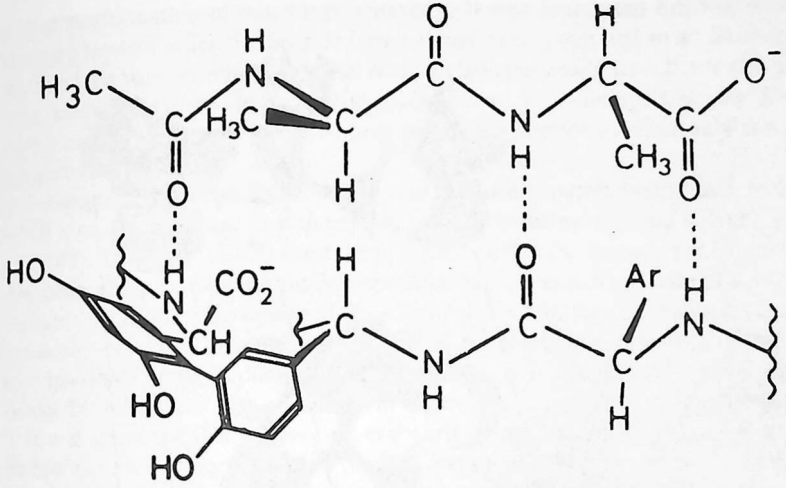


Figure 6 Illustration of some hydrogen-bonded interactions between Ac-D-Ala-D-Ala and a portion of the vancomycin molecule.

broadened signals were observed (44) because the chemical shifts of bound and free protons are time-averaged unless the solution is cooled.

The NMR studies showed (22) that two more hydrogen-bonded interactions were involved than had been postulated from models for vancomycin. An exploded view of the binding model for ristocetin is given in Figure 7. It should be noted that this is not exactly as given in the reference cited (22), because the change from *S* to *R* configuration has been made for the N-terminus in Figure 7 (as required from the evidence mentioned in the Structure section). The additional binding now proposed for ristocetin was that due to two more hydrogen bonds between the carboxyl group of the peptide and N*H*s of the antibiotic.

Apart from the chemical shift, an important effect exploited in these binding studies was, and continues to be, the negative nuclear Overhauser effect (nOe). An nOe occurs when irradiation of one proton at its resonant frequency causes a change in intensity of the signal due to another proton. Positive nOes occur for small molecules in a medium of low viscosity; negative nOes occur for large molecules (like ristocetin), particularly in a viscous solvent (like dimethyl sulfoxide). The presence of an nOe depends on the two protons involved being close to one another in space, hence the use of this effect in studies of binding. In the work referred to above, the nOe was used in a qualitative way: observation of a decrease in signal intensity meant two protons were close in space but no inter-proton distances were derived. More quantitative uses of the nOe will be described below.

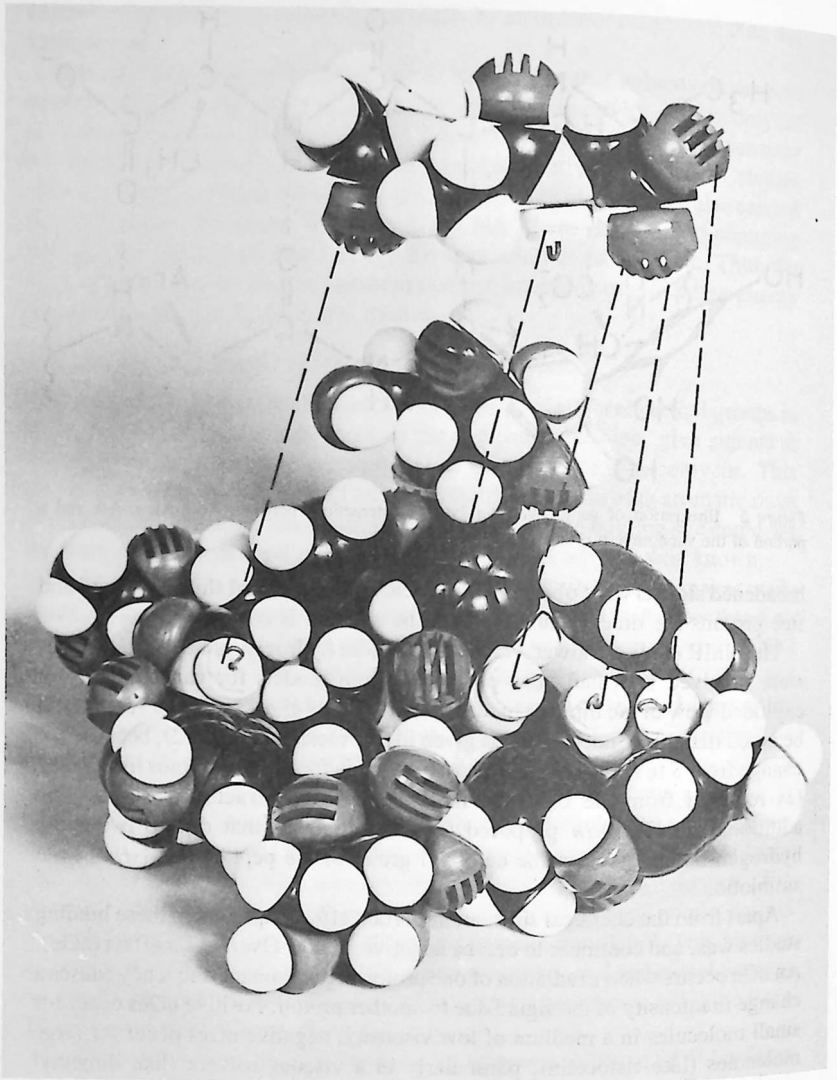


Figure 7 Exploded view (using CPK models) of the binding between Ac-D-Ala-D-Ala and ristocetin. Hydrogen bonds are shown by dashed lines.

At this stage, further studies of vancomycin binding to several peptide analogs were made using chemical shifts as the main tool (6). From studies of the pH dependence of the binding, it was deduced that a conformational change occurs upon binding so that the cystal structure of CDP-I does not represent the

bound conformation of vancomycin exactly. It was found that binding was strongly favored when the peptide terminal carboxyl group was in an anionic state and the vancomycin N-terminal *N*-methylleucine was in its cationic state. This meant that these groups must be important for binding; thus the *N*-methylamino group was proposed to approach the peptide more closely than suggested by the CDP-I model.

Subsequently, it was found possible to produce separate bound and free NMR signals for a vancomycin:peptide mixture by using a mixed solvent at 0°C (43). Using the results from the previous studies on vancomycin (6) and ristocetin (22), the binding model for vancomycin was further refined using chemical shift and nOe evidence. In summary, on binding, the vancomycin molecule—which is much more flexible in the right-hand half than is ristocetin—folds up to produce a binding site very similar to the one shown for ristocetin in Figure 7. This is shown in Figure 8 in a similar exploded view.

It was proposed that because several parts of the molecule become more mobile (i.e. have more entropy) on dissociation of the vancomycin:peptide complex, this process was more favorable than that for ristocetin. This effect may account, at least in part, for the fact that the free energy of activation is lower for dissociation of the vancomycin complex ($\Delta G\ddagger \sim 55$ kJ mol^{-1}) than for the ristocetin complex ($\Delta G\ddagger \sim 75$ kJ mol^{-1}), and it may account for the noted difference in exchange rates of free and bound components (as seen from the behavior of NMR signals described above).

The most recent and detailed examination of the binding sites in vancomycin and ristocetin has used a more refined cell-wall analog, namely Ac_2-L-Lys-D-Ala-D-Ala, as well as Ac-D-Ala-D-Ala. In this study (47), nOes were used in a more quantitative way. [This technique was also used to examine the stereochemistry of unbound vancomycin (48)].

Theory shows that the nOe (i.e. change in signal intensity of the affected proton) grows in an exponential way as the first proton is irradiated. The rate of growth, characterized by a half-life, $t_{1/2}$, is directly related to the distance, r, between the two protons. Thus $t_{1/2}$ is proportional to the time-averaged value of r^{-6}, and therefore the derived values of r represent lower limits for the inter-proton distances as they are weighted by the sixth-power term. These nOes are most easily observed using difference spectra (39) that are formed by the subtraction of two spectra; one is obtained with irradiation of a chosen proton, another with irradiation at a frequency where no protons in the system resonate.

It was concluded from these studies that hydrophobic interactions between peptide methyl groups and the hydrocarbon elements of the antibiotic help to strengthen the binding, as intermolecular nOes (i.e. those between protons of cell-wall analog and protons of antibiotic), together with intramolecular nOes, indicate proximity of such fragments. In order to obtain approximate intermolecular proton-proton distances in Angstroms (Å) from $t_{1/2}$ values, it was

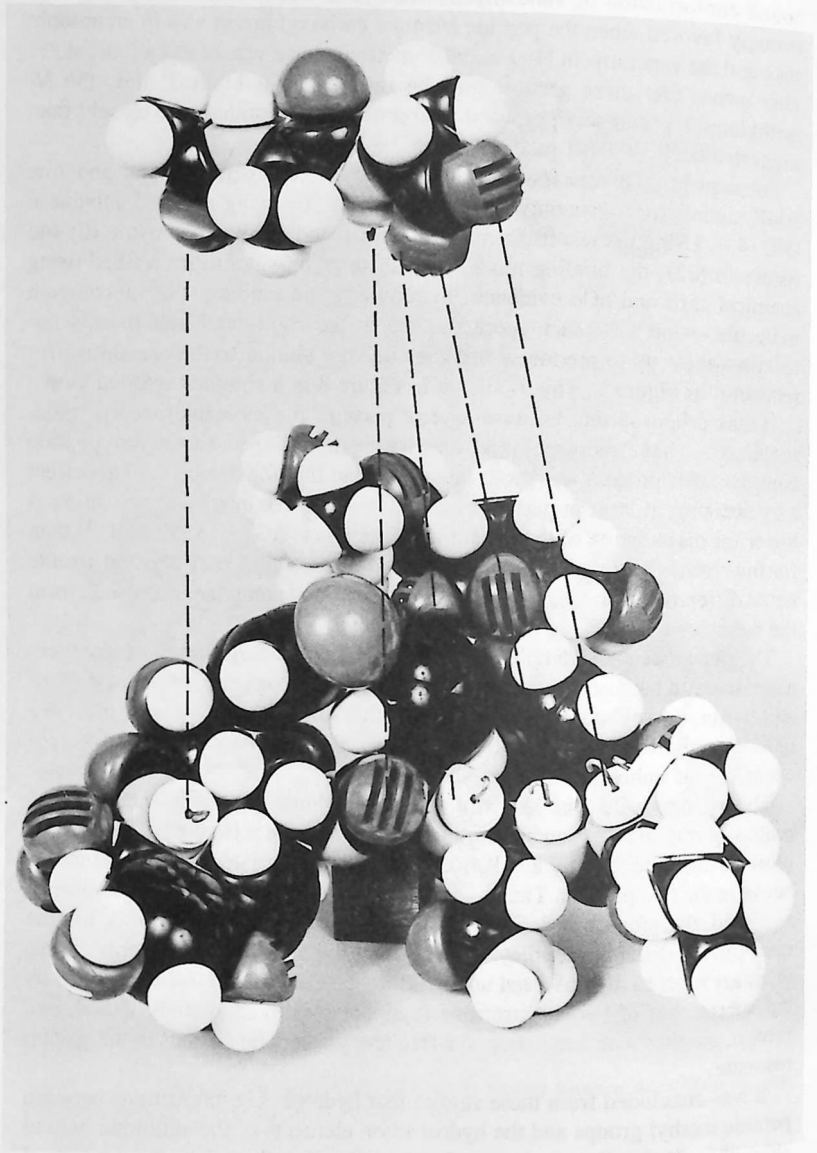


Figure 8 Exploded view of the binding between Ac-D-Ala-D-Ala and vancomycin. Hydrogen bonds are shown by dashed lines.

necessary to perform a calibration using two *ortho* aromatic protons (of known spatial separation). It was found that the alanine protons of the tripeptide cell-wall analog were typically 2–3 Ångstroms distant from certain aromatic and sugar protons in ristocetin. This is the range expected for a "tight" binding site. However, the lysine moiety did not give many rapidly growing nOes to the antibiotic, and it was concluded that this residue is not strongly bound. Following the earlier work of Perkins and coworkers, which showed that the D-Ala-D-Ala moiety is the most important structural component in binding, this finding was not unexpected. Thus the earlier proposals (6, 43) that vancomycin folds up on binding were confirmed by the measurement of the time-dependence of nOes. It was also found that the aromatic ring of the N-terminal residue (ring IV of Figure 3) in ristocetin folded in, presumably to increase hydrophobic interactions.

Relatively recently, avoparcin (10) and antibiotic A35512B (21) also have been shown to form complexes with Ac-D-Ala-D-Ala. This confirms that not only do all the antibiotics of the group so far studied display the same biochemical behavior (inhibition of bacterial cell-wall synthesis) but that the molecular basis of the antibiotic action is undoubtably the same in all cases. As suggested in the Structure section, this is because all the peptides have aglycones with relatively similar structures.

Role of the Sugars

Although a detailed picture of the binding sites of the antibiotics appears to have been obtained, many features are not understood. For example, in the case of in vitro studies, no detailed explanation of the orders of magnitude of the binding constants for the various cell-wall analogs (36) can be given. In vivo, more fundamental problems emerge. It was mentioned above that aglucovancomycin retained about three fourths of the biological activity of vancomycin (27). Ristocetin, by contrast, is not as active as its pseudoaglycone (ψ-aglycone), which is produced by removing all sugars except the ristosamine (30, 37) (R_9 in Figure 1). Although the sugars are clearly advantageous in promoting aqueous solubility of the antibiotics, their effects on antibiotic activity are not clearly understood.

Moreover, although the ψ-aglycone of ristocetin does not produce the blood platelet aggregation found for the intact antibiotic (24), it was found to be more toxic than ristocetin A when administered to mice or rats intravenously (30). No explanation for this increased toxicity has been given. It appears likely, however, that it is the polysaccharide unit of ristocetin that, possibly by simulating the presence of epithelial polysaccharide exposed in a wounding process, causes (in conjunction with other factors) blood platelet aggregation.

SUMMARY

It has been shown that after more than two decades of work on the structure and mode of action of the vancomycin group of antibiotics we have quite a detailed picture of aspects of their binding in vitro. Our introduction has indicated that these antibiotics are of continuing practical importance, whether in clinical use or animal husbandry. It is clear, however, that if one desires to modify these compounds to produce more effective antibiotic agents, more work is required—particularly to understand better the role of the sugar moieties. Nevertheless, with our present knowledge of the structure and mode of action of the antibiotics we can be fairly confident that they represent a group of compounds specifically evolved to aid the survival of the producing organisms by killing or inactivating competing gram-positive bacteria.

Literature Cited

1. Bogan, J. A. 1978. *Medicamentos de Actualidad* 14:42
2. Brazhnikova, M. G., Lomakina, N. N., Lavrova, M. F., Tolsztyth, I. V., Yurina, M. Sz., Klyueva, L. M. 1963. *Antibiotiki* 8:392–96
3. Brown, J. P., Feeney, J., Burgen, A. S. V. 1975. *Mol. Pharmacol.* 11:119–25
4. Brown, J. P., Terenius, L., Feeney, J., Burgen, A. S. V. 1975. *Mol. Pharmacol.* 11:126–32
5. Chatterjee, A. N., Perkins, H. R. 1966. *Biochem. Biophys. Res. Commun.* 24: 489–94
6. Convert, O., Bongini, A., Feeney, J. 1980. *J. Chem. Soc. Perkin Trans. 2* 1980:1262–70
7. Debono, M., Merkel, K. E., Weeks, R. E., Cole, H. J. 1982. *US Patent 4,322,406*; and references therein
8. Fainstein, V., LeBlanc, B., Bodey, G. P. 1983. *Antimicrob. Agents Chemother.* 23:497–99
9. Farber, B. F., Moellering, R. C. 1983. *Antimicrob. Agents Chemother.* 23:138–41
10. Fesik, S. W., Armitage, I. M., Ellestad, G. A., McGahren, W. J. 1982. *184th Natl. Meet. Am. Chem. Soc., Kansas City,* Abstr. MEDI 66. Washington DC: Am. Chem. Soc.
11. Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., Waring, M. J. 1981. *The Molecular Basis of Antibiotic Action*. London: Wiley. 646 pp. 2nd ed.
12. Geraci, J. E., Hermans, P. E. 1983. *Mayo Clin. Proc.* 58:88–91
13. Ghuysen, J. M. 1980. *Top. Antibiot. Chem.* 5(Part A):9–118
14. Grundy, W. E., Sinclair, A. C., Theriault, R. J., Goldstein, A. W., Rickher, C. J., et al. 1957. *Antibiot. Ann.* 1956–1957:687–92
15. Hammond, S. J., Williams, D. H., Nielsen, R. V. 1983. *J. Chem. Soc. Chem. Commun.* 1983:116–17
16. Hammond, S. J., Williamson, M. P., Williams, D. H., Boeck, L. D., Marconi, G. G. 1982. *J. Chem. Soc. Chem. Commun.* 1982:344–46
17. Harris, C. M., Harris, T. M. 1982. *J. Am. Chem. Soc.* 104:4293–95
18. Harris, C. M., Harris, T. M. 1982. *J. Am. Chem. Soc.* 104:365–67
19. Howard, M. A., Firkin, B. G. 1971. *Thromb. Diath. Haemorrh.* 26:362–69
20. Hunt, A. H. 1983. *J. Am. Chem. Soc.* 105:4463–68
21. Hunt, A. H., Vernon, P. D. 1981. *J. Antibiot.* 34:469–71
22. Kalman, J. R., Williams, D. H. 1980. *J. Am. Chem. Soc.* 102:906–12
23. Kunstmann, M. P., Mitscher, L. A., Porter, J. M., Shay, A. J., Darken, M. A. 1969. *Antimicrob. Agents Chemother.* 1968:242–45
24. Kuwahara, St. S., Chambers, W. T. 1978. *Experientia* 34:532–34
25. Lomakina, N. N., Zenkova, V. A., Bognár, R., Sztaricskai, F., Sheinker, Yu. N. Turchin, K. F. 1968. *Antibiotiki* 13:675–82
26. Lowy, F. D., Chang, D. S., Lash, P. R. 1983. *Antimicrob. Agents Chemother.* 23:932–34
27. Marshall, F. J. 1965. *J. Med. Chem.* 8:18–22
28. McCormick, M. H., Stark, W. M., Pittenger, G. E., Pittenger, R. C., McGuire, J. M. 1956. *Antibiot. Ann.* 1955–56:606–11
29. Michel, K., Shah, R. M., Hamill, R. L. 1977. *Antimicrob. Agents Chemother., 17th Conf., New York,* Abstr. 41
30. Nielsen, R. V., Hyldig-Nielsen, F., Jacobsen, K. 1982. *J. Antibiot.* 35:1561–64
31. Nieto, M., Perkins, H. R. 1971. *Biochem. J.* 123:789–803
32. Nieto, M., Perkins, H. R. 1971. *Biochem. J.* 123:773–87
33. Nieto, M., Perkins, H. R. 1971. *Biochem. J.* 124:845–52
34. Parenti, F., Beretta, G., Berti, M., Arioli, V. 1978. *J. Antibiot.* 31:276–83
35. Perkins, H. R. 1969. *Biochem. J.* 111:195–205
36. Perkins, H. R. 1982. *Pharmac. Ther.* 16:181–97
37. Philip, J. E., Schenck, J. R., Hargie, M. P., Holper, J. C., Grundy, W. E. 1961. *Antimicrob. Agents Ann.* 1960:10–16
38. Rogers, H. J., Perkins, H. R., Ward, J. B. 1980. *Microbial Cell Walls and Membranes*. London: Chapman & Hall. 564 pp.
39. Sanders, J. K. M., Mersh, J. D. 1983. *Prog. Nucl. Mag. Res. Spec.* 15:353–400
40. Sheldrick, G. M., Jones, P. G., Kennard, O., Williams, D. H., Smith G. A. 1978. *Nature* 271:223–25
41. Shorin, V. A., Yudintsev, S. D., Kunrat, I. A., Gol'dberg, L. E., Pevsner, N. S., et al. 1957. *Antibiotiki* 2:44–49
42. Sinha, R. K., Neuhaus, F. C. 1968. *J. Bacteriol.* 96:374–82
43. Williams, D. H., Butcher, D. W. 1981. *J. Am. Chem. Soc.* 103:5697–700
44. Williams, D. H., Kalman, J. R. 1977. *J. Am. Chem. Soc.* 99:2768–74
45. Williams, D. H., Rajananda, V., Bojesen, G., Williamson, M. P. 1979. *J. Chem. Soc. Chem. Commun.*, 906–8
46. Williams, D. H., Rajananda, V., Williamson, M. P., Bojesen, G. 1980. *Top. Antibiot. Chem.*, 5:(Part B)119–58
47. Williams, D. H., Williamson, M. P., Butcher, D. W., Hammond, S. J. 1983. *J. Am. Chem. Soc.* 105:1332–39
48. Williamson, M. P., Williams, D. H. 1981. *J. Am. Chem. Soc.* 103:6580–85

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ALTERNATIVE PATHWAYS OF CARBOHYDRATE UTILIZATION IN PSEUDOMONADS

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AIMS

The aim of this review is to assess the relative importance of different pathways of carbohydrate utilization in pseudomonads—based largely on recent information about the growth behavior of mutants deficient in pathway enzymes. Taken in conjunction with data on patterns of enzyme induction in the presence of specific carbohydrates, the mutant analyses provide a coherent view of the main routes of carbohydrate dissimilation in this important group of bacteria.

The variety of mutants now available has made it possible to identify alternative pathways whose contribution had not been appreciated. This new information has indicated the need for reinterpretation of the roles of the Entner-Doudoroff, Embden-Meyerhof, and pentose-shunt pathways as conceived from early radiorespirometric data (102, 127, 129). To some extent the review reflects our own bias towards investigation of *P. aeruginosa* [the pseudomonad most amenable to genetic analysis (94, 118)] and *P. cepacia* [the most nutritionally versatile of the pseudomonads (7)].

Recent progress in the genetic analysis of pseudomonads, most conspicuously of *Pseudomonas aeruginosa* (52, 56, 57, 118), promises to make these bacteria highly suitable for studies of the regulation of gene expression. In view of the unusual catabolic potential of these organisms, it can be expected that such studies will reveal novel mechanisms of control of catabolic pathways. We summarize here current data on the organization of genes related to carbohydrate utilization in *P. aeruginosa*.

We hope through this review to define areas of investigation whose pursuit is likely to extend our understanding of the regulation of catabolic processes in this nutritionally versatile group of microorganisms. Two excellent reviews on overall aspects of carbohydrate metabolism in microorganisms that have guided our efforts are those of Anderson & Wood (4) and of Fraenkel & Vinopal (49).

ALTERNATIVE ROUTES OF CONVERSION OF GLUCOSE TO 6-PHOSPHOGLUCONATE

Degradation of glucose by pseudomonads requires its conversion to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway (45, 62). 6-Phosphogluconate is a key intermediate in this process. Pseudomonads do not metabolize glucose to triose phosphate via the Embden-Meyerhof pathway, because they are devoid of 6-phosphofructokinase (54, 95, 120, 135, 151). There are two routes of conversion of glucose to 6-phosphogluconate: the direct oxidative pathway, which acts on glucose extracellularly (59, 67, 85, 109, 112, 139, 140, 146), and the phosphorylative pathway (14, 42, 54, 59, 68, 69, 95, 104, 107, 135, 146), which does so intracellularly. The two pathways are outlined in Figure 1. As is described below they represent truly alternative routes of 6-phosphogluconate formation, with operation of one able to compensate for loss of the other. However, depending upon the physiological conditions, one or the other ordinarily predominates.

Early studies of glucose dissimilation by pseudomonads (37, 71, 133) indicated that these bacteria oxidized glucose to D-gluconic and 2-keto-D-gluconic acid. These reactions were subsequently shown to represent the initial stages of conversion of glucose to 6-phosphogluconate (6PGA) via the direct oxidative pathway in which gluconate, 2-ketogluconate, and 2-keto-6-phosphogluconate are intermediates (85, 89, 112). The two successive oxidations required to convert glucose to 2-ketogluconate are carried out in the periplasm by membrane-associated, pyridine nucleotide–independent, glucose and gluconate dehydrogenases (77–79). 2-Ketogluconate is transported into the cell (39, 72, 147, 148) and then acted upon by an ATP-dependent kinase that catalyzes the formation of 2-keto-6-phosphogluconate (89, 91, 112). The last step, conversion of 2-keto-6-phosphogluconate to 6-phosphogluconate, requires expenditure of NAD(P)H (50, 89, 112). To our knowledge the complement of enzymes comprising the segment of this pathway beyond 2-ketogluconate has been demonstrated in all pseudomonads in which its presence has been tested. *P. acidovorans* and *P. citronellolis* both appear to differ from other pseudomonads by lacking activity of glucose and gluconate dehydrogenases (95, 145).

Most pseudomonads also have the potential to accumulate glucose by active transport and convert it to 6-phosphogluconate by ATP-dependent phosphorylation to glucose-6-phosphate and pyridine nucleotide–dependent oxidation of glucose-6-phosphate (26, 27, 42, 51, 59, 68, 69, 72, 86, 95, 135, 147, 151). This set of reactions comprises the phosphorylative pathway. *P. acidovorans* is an exception; it lacks glucokinase as well as glucose dehydrogenase activity and therefore fails to utilize glucose (145).

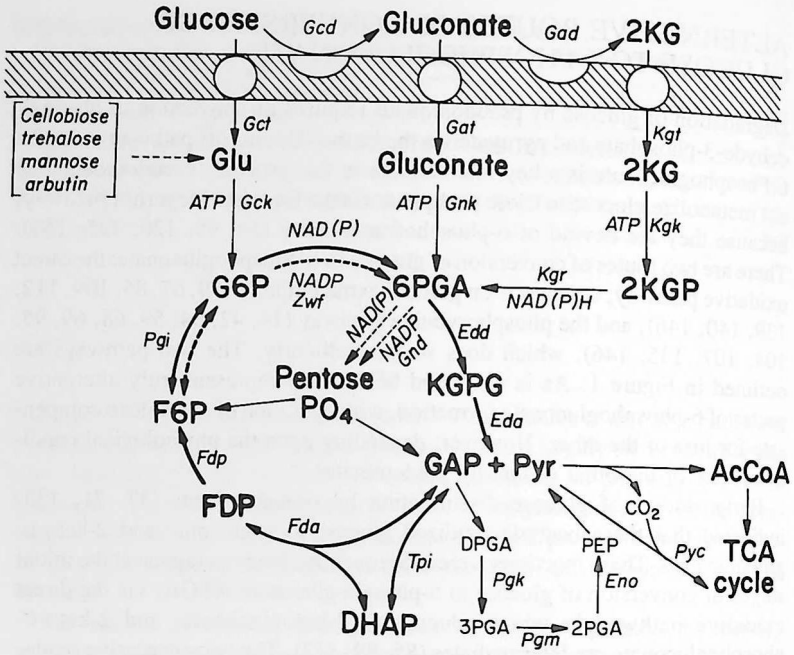


Figure 1 Enzymes related to glucose utilization in pseudomonads. *Dashed lines* indicate features characteristic of *P. cepacia*. All other reactions shown have been demonstrated in *P. aeruginosa*. Sugars and intermediates are of the D-configuration. Abbreviations: Gcd and Gad, membrane associated glucose and gluconate dehydrogenases; Gct, Gat, and Kgt and Gck, Gnk, and Kgk represent, respectively, transport systems and ATP-dependent kinases for glucose, gluconate, and 2-ketogluconate; Kgr, 2-keto-6-phosphogluconate reductase; Zwf and Gnd, glucose-6-phosphate (G6P) and 6-phosphogluconate (6PGA) dehydrogenases; Edd and Eda, 6PGA dehydratase and KDPG aldolase. Recycling of glyceraldehyde-3-phosphate (GAP) into the Entner-Doudoroff pathway involves: Tpi, triose phosphate isomerase; Fda, fructose 1,6-diphosphate aldolase; Fdp, fructose 1,6-diphosphatase; and Pgi, phosphoglucoisomerase; Pyc refers to pyruvate carboxylase. For other abbreviations see Figure 2.

Mutants Blocked in the Direct Oxidative Route

The simplest procedure for obtaining mutants in which the direct oxidative route fails to operate is to screen for a block at the first step of the pathway. Glucose dehydrogenase–deficient (Gcd^-) strains are readily distinguished on indicator plates as colonies that fail to form acid from glucose (15, 67, 85, 109, 132). With bromthymol blue as pH indicator, Gcd^- strains lack the yellow halo characteristic of acid-producing wild-type colonies. With eosin and methylene blue (EMB plates), Gcd^- strains appear white to pink as compared to dark purple for wild-type bacteria. Certain pseudomonads such as *P. cepacia* and *P. fluorescens* also exhibit a bright green sheen (15, 67, 109).

Glucose dehydrogenase–deficient mutants have been isolated in *Pseudomonas aeruginosa* (85, 132), *P. fluorescens* (109), *P. putida* (139), and *P. cepacia* (15, 67). The *P. aeruginosa* and *P. cepacia* strains still grew on glucose despite the block in the direct oxidative pathway. *P. cepacia* compensated for the loss of direct oxidative pathway activity by expanding the phosphorylative pathway (14). In contrast, loss of glucose dehydrogenase activity in *P. putida* resulted in failure to utilize glucose (139).

Comparable mutant studies specifically implicating gluconate dehydrogenase in dissimilation of glucose and gluconate have not been reported. This is largely due to the lack of specific selection procedures suitable for isolation of gluconate dehydrogenase–deficient (Gad^-) strains. Berka & Lessie isolated *P. cepacia* mutants deficient in both glucose and gluconate dehydrogenase activity (14a). These strains still utilized gluconate, suggesting that the direct conversion of gluconate to 6-phosphogluconate (see Figure 1) is a potentially significant pathway.

Vicente & Canovas isolated a mutant blocked in the fourth step of the direct oxidative pathway: phosphorylation of 2-ketogluconate (139). This strain, Y-71.4, grew extremely slowly on glucose or gluconate and failed to utilize 2-ketogluconate. The growth behavior of strain Y-71.4 suggested that the uptake and direct phosphorylation of gluconate was not physiologically important for gluconate utilization in *P. putida*. Another strain, Y-13.10, possibly a 2-ketogluconate reductase–deficient strain, had a phenotype like that of strain Y-71.4, but possessed normal levels of 2-ketogluconokinase.

Influence of O_2, Glucose Concentration, and Temperature on Operation of the Direct Oxidative Pathway

When *P. aeruginosa* was grown anaerobically in media containing glucose with nitrate as electron acceptor, the bacteria were devoid of glucose dehydrogenase (58, 59). The absence of this activity was confirmed by demonstrating that the bacteria failed to form gluconate or 2-ketogluconate from radiolabeled glucose (58). Growth on glucose was not precluded by the absence of glucose dehydrogenase, because the phosphorylative pathway was intact and able to operate under these conditions. The importance of the phosphorylative pathway in utilization of glucose under conditions of anaerobic respiration was confirmed by showing that glucose utilization was blocked in a Zwf^- mutant (59). Glucose utilization was not affected when the same mutant was grown under aerobic conditions. The results demonstrate that the phosphorylative pathway is essential for utilization of glucose under conditions of anaerobic respiration and that the direct oxidative pathway can substitute for the phosphorylative pathway under aerobic conditions.

Mitchell & Dawes examined the influence of oxygen limitation on enzymes

of the direct oxidative and phosphorylative pathways of glucose utilization in chemostat cultures of *P. aeruginosa* (86). Their results were consistent with the findings of Hunt & Phibbs. The specific activities of enzymes of the direct oxidative pathway decreased at least five-fold following a shift from conditions of nitrogen limitation to oxygen limitation.

Whiting et al examined the levels of enzymes of the direct oxidative and phosphorylative pathways in chemostat cultures of *P. aeruginosa* PAO1 grown with either glucose or nitrogen as limiting nutrient (147, 148). Under conditions in which glucose was limiting, glucose-transport activity and the levels of phosphorylative pathway enzymes were increased preferentially relative to levels of direct oxidative pathway enzymes. The authors interpreted the results as indicating that at low concentration, glucose is metabolized primarily via the phosphorylative route, whereas glucose provided at high concentration is metabolized primarily via the direct oxidative route.

Lynch et al compared the levels of direct oxidative and phosphorylative pathway enzymes and the extent accumulation of extracellular 2-ketogluconate in cultures of the facultative psychrophile *P. fluorescens* E20 grown on glucose at 5°C and at 30°C (72–75). These investigators observed that the pathway of glucose dissimilation shifted with increasing growth temperature from the direct oxidative to the phosphorylative route. This conclusion was consistent with their observations that growth in the presence of glucose induced glucose dehydrogenase at 5°C but not at 30°C, in parallel with the pattern of accumulation of 2-ketogluconate (74, 75). The bacteria grew more slowly on 2-ketogluconate than on glucose or gluconate, suggesting that transport of 2-ketogluconate might be the limiting step in dissimilation of glucose via the direct oxidative pathway. Palumbo & Witter (101) reported that the yield of bacteria during growth of *P. fluorescens* in chemostat cultures decreased as the temperature was decreased, possibly due to less efficient utilization of ketogluconate at lower temperatures.

Cellular Location and Other Characteristics of Glucose and Gluconate Dehydrogenases

Glucose and gluconate dehydrogenase have been reported to be membrane-associated in all pseudomonads where their distribution in different cell fractions has been examined (15, 23, 37, 53, 59, 67, 77–79, 139). There is some question about whether significant levels of either enzyme exist free in the cytoplasm (139). In view of the ease with which these enzymes can be released from membranes (15, 77–79), it must be considered that recovery of these activities in the nonsedimentable fraction of disrupted cell suspensions represents an artifact of cell disintegration.

Noting that suspensions of *P. aeruginosa* convert glucose to 2-ketogluconate more rapidly than they transport carbon from glucose into the cell (71, 133), Midgley & Dawes proposed that glucose and gluconate dehydrogenases are situated in the membrane in such a way as to permit their action on glucose and gluconate in the periplasm (85). This is consistent with other findings indicating that growth of bacteria on glucose is accompanied by the induction of an active transport system for the uptake of 2-ketogluconate (39, 89, 147, 148). Dawes and his coworkers have suggested that one advantage of rapid conversion of glucose to 2-ketogluconate might be the sequestration of carbon source in a form not as readily utilized by competing bacteria as glucose (147, 148).

Glucose and gluconate dehydrogenases have been purified and characterized with respect to substrate specificity, cofactor requirement, and other properties (77–79). The enzymes are released from membranes in active form following treatment with detergents such as Triton X-100 (15, 77–79). Glucose dehydrogenase from *P. fluorescens* was a quinoprotein (77). It was comprised of a single peptide chain with a molecular weight of about 90,000. Its activity with D-galactose was less than 10% of that with D-glucose. In contrast, the D-glucose dehydrogenase from *P. cepacia,* which had a molecular weight of about 100,000, was as active with galactose as with glucose (15). D-gluconate dehydrogenases from *P. fluorescens* and *P. aeruginosa* were found to be flavoproteins with molecular weights of about 140,000 (78, 79). Both enzymes were comprised of three subunits of 70,000, 50,000, and 20,000 daltons.

Mutants Blocked in the Phosphorylative Route

Glucose-6-phosphate dehydrogenase–deficient (Zwf^-) mutants of *P. aeruginosa* were isolated among strains that had lost the capacity to utilize mannitol as sole carbon source (8, 107). The Zwf^- strains were unable to utilize glucose under conditions of anaerobic respiration but grew normally under aerobic conditions, where the direct oxidative pathway substituted for loss of phosphorylative pathway activity (59).

A number of mutants have been isolated that further confirm the importance of the phosphorylative pathway in glucose utilization. Allenza et al isolated a glucokinase-deficient (Glk^-) derivative of *P. cepacia* from a strain already deficient in glucose dehydrogenase by selection for loss of ability to utilize glucose (2, 14). When these Glk^- Gcd^- mutants reverted to growth on glucose, they regained glucokinase activity. The results confirm the participation of glucokinase as an integral component of the phosphorylative pathway of glucose utilization.

Stinson and his coworkers have defined the role in glucose transport of a 45,000-M_r binding protein that was released from *P. aeruginosa* by osmotic shock (131). Mutants deficient in this protein were selected from a Gcd^- derivative of ATCC strain 15692 by selection for loss of glucose chemotaxis and subsequent screening for bacteria able to utilize gluconate but unable to

utilize glucose (132). Feary et al isolated similar derivatives of *P. aeruginosa* PAO from a strain already blocked in the direct oxidative pathway (46).

A number of investigators have examined glucose uptake by intact cells and vesicle preparations (51, 72, 85, 88, 116, 117, 130, 141, 142, 147, 148). Midgley & Dawes distinguished two components of glucose uptake in *P. aeruginosa* (85). One, which was identical to that responsible for uptake of methylglucoside, was active at low concentrations of glucose and presumably is the only system promoting direct uptake of glucose. This system was repressed and inhibited by gluconate. Methylglucoside was accumulated but not metabolized by the cells. The other component of glucose uptake depended upon glucose and gluconate dehydrogenase–mediated formation of 2-ketogluconate and transport of the latter compound by a system distinct from that involved in uptake of methylglucoside. The apparent affinity of the authentic transport system was approximately 300-fold greater for glucose than for methylglucoside.

Different Patterns of Induction of Direct Oxidative and Phosphorylative Pathway Enzymes

Examination of the patterns of induction of enzymes related to glucose utilization in mutants of the type described above or in wild-type bacteria grown on different carbon sources indicated that synthesis or exogenous addition of gluconate or 2-ketogluconate was required to elicit formation of the full complement of direct oxidative pathway enzymes (14, 15, 58, 59, 67). This clearly was not a condition for induction of components of either the phosphorylative or Entner-Doudoroff pathways.

In *P. aeruginosa* PAO and OSU-64, gluconate but not glucose induced formation of gluconate dehydrogenase under conditions of anaerobic respiration (58, 59). The capacity to form gluconate dehydrogenase during growth in the presence of glucose was restored under aerobic conditions, coincident with the reappearance of glucose dehydrogenase.

In *P. cepacia*, which forms glucose dehydrogenase constitutively, glucose triggered formation of subsequent enzymes of the direct oxidative pathway because of its rapid conversion to gluconate (14, 67). In Gcd^- mutants, glucose failed to elicit formation of gluconate dehydrogenase and of a 6-phosphogluconate dehydrogenase active with NAD, whose synthesis was regulated coordinately with enzymes of the direct oxidative pathway (14). In the presence of gluconate, induction of these enzymes was normal in the Gcd^- strains. In one class of mutants, which lacked both glucose and gluconate dehydrogenase activities, 2-ketogluconate but not gluconate induced the 6-phosphogluconate dehydrogenase active with NAD, suggesting that 2-ketogluconate may be the physiological inducer of the direct oxidative pathway enzymes (14a).

When *P. cepacia* was grown on mannitol, sorbitol, or fructose, the only direct oxidative pathway enzyme detected was glucose dehydrogenase. Under these conditions the 6-phosphogluconate dehydrogenase active with NAD was not formed. A similar failure to induce enzymes of the direct oxidative pathway was noted during growth of *P. cepacia* on disaccharides such as cellobiose or trehalose, or on aromatic conjugates of glucose such as arbutin and salicin (14). Utilization of these compounds was blocked in glucokinase-deficient strains, suggesting that after entry into the cells they are converted to glucose and metabolized via the phosphorylative pathway (see Figure 1). Their failure to induce direct oxidative pathway enzymes is consistent with the idea that conversion of glucose to gluconate and 2-ketogluconate occurs only extracellularly. Levels of glucokinase and glucose-6-phosphate dehydrogenase and capacity to transport glucose were four-fold higher in bacteria grown on cellobiose, trehalose, arbutin, salicin, or mannose than in bacteria grown on glucose. Mannose appeared to be metabolized to glucose-6-phosphate via two routes (1, 14). One involved its conversion to glucose by mannose epimerase and the other its conversion to fructose by mannose isomerase. A similar hyperinduction of phosphorylative pathway enzymes was observed during growth of Gcd^- mutants on glucose. The results suggest that hyperinduction of phosphorylative pathway enzymes is a consequence of intracellular accumulation of glucose.

CENTRAL ROLE OF THE ENTNER-DOUDOROFF PATHWAY

The Entner-Doudoroff (ED) pathway was first elucidated in *P. saccharophila* (37, 45). Radiorespirometric experiments subsequently indicated that this also represented the major route of glucose utilization in other pseudomonads (102, 127, 129). More recently the Entner-Doudoroff pathway has been demonstrated to play an important role in degradation of gluconate in enteric bacteria (43, 49). 6-Phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, the two enzymes comprising the pathway (see Figure 1), have been characterized extensively in *P. putida* by W. A. Wood and his coworkers (64, 65, 83, 84). Kersters & De Ley have examined the distribution of these enzymes in a variety of gram-negative bacteria (61, 62).

Mutants Deficient in 6-Phosphogluconate (6PGA) Dehydratase and 2-Keto-3-deoxy-6-Phosphogluconate (KDPG) Aldolase

Mutants deficient in activities of the two Entner-Doudoroff pathway enzymes have been isolated in *P. putida* (139), *P. aeruginosa* (8, 18, 54, 115), *P. cepacia* (3), *Hydrogenomonas eutropha* (20), and *Escherichia coli* (49). In the

aforementioned pseudomonads, mutants blocked in the ED pathway were unable to utilize glucose or gluconate. This was in marked contrast to the behavior of the *E. coli* mutants, which grew normally on glucose and retained some capacity to utilize gluconate via the pentose shunt.

Failure of 6-phosphogluconate dehydratase–deficient (Edd^-) and KDPG aldolase–deficient (Eda^-) strains of the aforementioned bacteria to utilize glucose or gluconate is consistent with radiorespirometric and enzyme-induction data implicating this pathway as the major route of glucose utilization in these bacteria (102, 127, 129). Entner-Doudoroff pathway mutants of *P. aeruginosa* failed to utilize mannitol and were seriously impaired in utilization of fructose and glycerol (8, 18, 115), pointing to a general involvement of the Entner-Doudoroff pathway in dissimilation of carbohydrates. *P. cepacia* Edd^- and Eda^- mutants failed to utilize most carbohydrates tested, including glucose, fructose, mannose, 2-ketogluconate, mannitol, sorbitol, galactitol, ribitol, cellobiose, and trehalose (3, 14). Deficiency of these enzymes also affected growth on galactose (15).

Failure of Edd^- and Eda^- mutants to utilize a particular carbohydrate should not necessarily be interpreted to indicate that its degradation proceeds exclusively via the Entner-Doudoroff pathway. As is noted below, the potential for hexose phosphate toxicity due to accumulation of 6PGA and/or KDPG must be taken into account before rejecting the possibility of alternative pathways (3).

The pattern of induction of the two Entner-Doudoroff pathway enzymes in both wild-type and mutant strains of several pseudomonads is distinct from that of enzymes of the direct oxidative pathway and appears to parallel that of glucose-6-phosphate dehydrogenase (3, 68, 69, 82, 107). Both 6-phosphogluconate dehydratase and KDPG aldolase were induced when *P. aeruginosa* was grown anaerobically with nitrate as electron acceptor and glucose as carbon source (59), or when glucose dehydrogenase–deficient strains of *P. cepacia* were grown on glucose (14, 67)—conditions under which enzymes of the direct oxidative pathway remain uninduced. This was also the case during growth of *P. aeruginosa* on mannitol, fructose, and glycerol (82, 107) or of *P. cepacia* on cellobiose, trehalose, or sorbitol (1, 2). In *P. aeruginosa,* induction of Enter-Doudoroff pathway enzymes was impaired in Zwf^- mutants grown on lactate or casamino acids in the presence of mannitol, fructose, or glycerol, but was normal in bacteria grown on glucose or gluconate (82, 107).

These results suggested that the actual inducer of the Entner-Doudoroff pathway enzymes might be 6-phosphogluconate. Both in *P. aeruginosa* and in *P. cepacia,* growth of Edd^- mutants in the presence of glucose and alternative carbon sources such as lactate, pyruvate, or citrate resulted in induction of KDPG aldolase (3, 18), consistent with the notion that 6-phosphogluconate is the physiological inducer of this enzyme. It should be noted that genes encoding glucose-6-phosphate dehydrogenase and the two Entner-Doudoroff pathway enzymes are tightly linked on the *P. aeruginosa* chromosome, raising the possibility that they are organized into a single operon (115).

A somewhat different pattern of induction of ED pathway enzymes has been reported for *P. putida,* where 6-phosphogluconate dehydratase and KDPG aldolase appeared not to be regulated coordinately (140). Mutants unable to metabolize gluconate and 2-ketogluconate were nevertheless induced with respect to formation of 6-phosphogluconate dehydratase when grown in the presence of these compounds. Furthermore, Edd^- mutants failed to produce KDPG aldolase when grown in the presence of these compounds. On the basis of these observations, Vincente & Canovas proposed that the physiological inducers of 6-phosphogluconate dehydratase and KDPG aldolase were, respectively, gluconate or 2-ketogluconate and KDPG.

Recycling of Glyceraldehyde-3-Phosphate via the Entner-Doudoroff Pathway

Two lines of evidence indicate that, in pseudomonads, glyceraldehyde-3-phosphate derived from the cleavage of KDPG during operation of the ED pathway is recycled to 6-phosphogluconate via fructose 1,6-diphosphate, fructose-6-phosphate, and glucose-6-phosphate, instead of being directly converted to pyruvate via phosphoenolpyruvate: (*a*) ED pathway and related enzymes such as glucose-6-phosphate dehydrogenase are induced during growth on compounds such as glycerol (5, 54, 60, 68, 69, 82) and galactose (15, 70) that are metabolized directly to glyceraldehyde-3-phosphate, as well as on fructose, which in most pseudomonads enters the proposed cyclic route at the level of fructose-1,6-diphosphate (107, 120, 138); (*b*) Mutants blocked in the Entner-Doudoroff pathway were impaired in growth on fructose (2, 8, 107, 120), glycerol (18, 54, 106) and galactose (15), whereas mutants lacking phosphoglycerate kinase (5), enolase, or pyruvate kinase (P. V. Phibbs, Jr., unpublished data) appeared to grow normally on these carbohydrates. The induction of glucose-6-phosphate dehydrogenase during growth on gluconate (54, 58, 59, 68, 69, 85, 107, 109, 112, 140), 2-ketogluconate (112, 140), or galactose (14a, 70) was also consistent with such a scheme.

The idea that glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, with which it is in equilibrium, are recycled through the Entner-Doudoroff route was first proposed by Hochster & Katznelson, based on their studies of glucose dissimilation in *Xanthomonas phaseoli* (55).

SIGNIFICANCE OF THE OXIDATIVE HEXOSEMONOPHOSPHATE (HMP) PATHWAY

As noted above, the Entner-Doudoroff pathway represents the primary route of 6-phosphogluconate dissimilation in pseudomonads. In certain but not all pseudomonads, significant amounts of 6-phosphogluconate also appear to be

diverted into the hexosemonophosphate (HMP) pathway. This is particularly true in the case of *P. cepacia* and the closely related bacterium *P. marginata*, where 6-phosphogluconate dehydrogenase (6PGAD) is a prominent enzyme of glucose and gluconate degradation (3, 66, 69). These two pseudomonads contain both constitutive and inducible 6PGAD species—the latter being formed only in the presence of glucose, gluconate, or 2-ketogluconate (14). The constitutive enzyme (120,000 M_r) was specific for NADP and presumably plays an important role in formation of NADPH to drive reductive steps in biosynthesis (66). The inducible enzyme (80,000 M_r) was active with NAD or NADP and may supply reduced pyridine nucleotide required to convert 2-keto-6-phosphogluconate to 6-phosphogluconate (14).

Alternatively, this enzyme might itself catalyze the reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate. Either role would be consistent with its coordinate regulation with gluconate dehydrogenase (14). Two species of 6-phosphogluconate dehydrogenase were also identified in *P. fluorescens* (134). The major one was purified and its product identified as D-ribulose-5-phosphate. The minor species was presumed to catalyze the reduction of 2-keto-6-phosphogluconate to 6-phosphogluconate.

An unexpected finding with Eda^- and Edd^- mutants of *P. cepacia* was that these strains failed to utilize glucose or gluconate despite the prominence of 6-phosphogluconate dehydrogenase in this bacterium (3). Derivatives of an Edd^- strain, which had higher levels of activity of the 6-phosphogluconate dehydrogenase active with NAD (Edd^- $GndR^-$ mutants), regained the ability to utilize gluconate—albeit at a slower than normal rate (1, 3). The behavior of these strains suggested that the failure of Edd^- and Eda^- mutants to utilize glucose or gluconate was not due to inability of the pentose shunt to function but rather to accumulation and toxicity of 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate brought about by the block in the Entner-Doudoroff pathway.

In this context, the restoration of growth in the $GndR^-$ mutants (which were able to form as much as four- to six-fold higher levels of 6-phosphogluconate dehydrogenase in the presence of gluconate) presumably was due to a decrease in the levels of toxic intermediates. It should be noted that the Edd^- $GndR^-$ mutants were still unable to utilize fructose, mannitol, sorbitol, cellobiose, or trehalose—substrates that are dissimilated via 6-phosphogluconate but not gluconate and that therefore fail to induce the 6-phosphogluconate dehydrogenase active with NAD (1, 2, 14).

In contrast to the situation in *P. cepacia*, the HMP pathway appears to play no role in 6-phosphogluconate dissimilation by *P. aeruginosa* (105). This is due to a natural deficiency of 6-phosphogluconate dehydrogenase. Earlier reports of the existence of this enzyme in *P. aeruginosa* can now be attributed to a coupled reaction in which glyceraldehyde-3-phosphate formed from 6-

phosphogluconate by the action of the two Entner-Doudoroff pathway enzymes, 6-phosphogluconate dehydratase and KDPG aldolase, is oxidized by GAP dehydrogenase to simulate 6-phosphogluconate–dependent formation of NADH or NADPH. This reaction only occurred in crude bacterial extracts containing both 6-phosphogluconate dehydratase and KDPG aldolase. It was inhibited by NaF and was not accompanied by release of $^{14}CO_2$ from C1-labeled glucose (105, 120). With a mutational block in the Entner-Doudoroff pathway, there was essentially total loss of this activity (18, 105, 120).

It is important to note that 6-phosphogluconate dehydrogenase activity has never been reported in fractionated extracts of *P. aeruginosa*, as would be expected were an authentic 6-phosphogluconate dehydrogenase present. Heath & Gaudy detected low levels of an apparent 6-phosphogluconate dehydrogenase activity in mutants blocked in the Entner-Doudoroff pathway (54). However, these investigators did not rule out the possibility that the apparent 6-phosphogluconate dehydrogenase activity was due to residual Entner-Doudoroff pathway activity.

Earlier respirometric data in which the pattern of release of $^{14}CO_2$ from differentially labeled glucose was examined were interpreted to indicate that the pentose shunt played a significant role in dissimilation of glucose (102, 127, 129). However, these investigators did not take into account the possibility that glyceraldehyde-3-phosphate derived by the cleavage of KDPG might be recycled into the Entner-Doudoroff pathway. This would account for a lower yield of labeled CO_2 from C4 as compared to C1-labeled glucose.

Other pseudomonads in which the HMP pathway appeared not to play a significant role included *P. saccharophila* (37, 48, 120), *P. putida* (110, 120, 139), *P. acidovorans* (120, 145), *P. maltophila* (120), and *P. doudoroffii* (11). It is uncertain at this point whether the discrepancy in the literature concerning the presence of 6-phosphogluconate dehydrogenase activity in different isolates of *P. fluorescens* (120, 134, 138) reflects genetic differences between strains.

DISSIMILATION OF FRUCTOSE AND RELATED HEXITOLS

Importance of PEP:Fructose 1-Phosphotransferase, 1-Phosphofructokinase, and Fructose 1,6-diphosphatase

Figure 2 outlines reactions related to fructose utilization in pseudomonads. A feature of fructose metabolism in most pseudomonads that distinguishes it from that of other carbohydrates is the transport of this sugar into the cell by vectorial phosphorylation (11, 41, 114, 120, 138). Fructose was the only carbohydrate

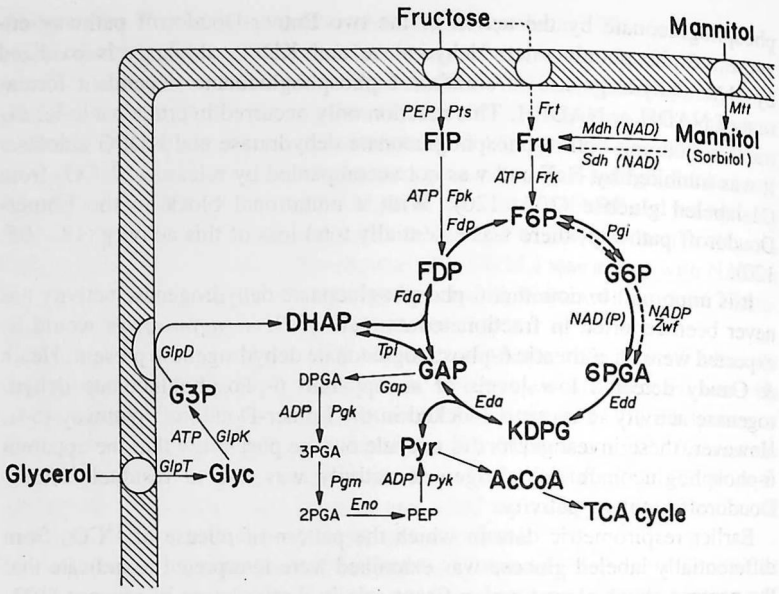


Figure 2 Enzymes related to utilization of glycerol, fructose, and related hexitols. *Dashed lines* indicate features characteristic of *P. cepacia*. All other reactions have been demonstrated in *P. aeruginosa*. Abbreviations: Pts, PEP:fructose phosphotransferase system; Fpk, 1-phosphofructokinase; GlpT, Frt, and Mtt, transport systems for glycerol, fructose, and mannitol; GlpK and Frk, ATP-dependent kinases for glycerol and fructose; GlpD, membrane-associated glycerol-3-phosphate dehydrogenase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Pgk, 3-phosphoglycerate kinase; Pgm, phosphoglyceromutase; Eno, enolase; Pyk, pyruvate kinase; Mdh and Sdh, mannitol and sorbitol dehydrogenases. For other abbreviations see Figure 1.

for which PEP-dependent phosphotransferase activity was demonstrated in pseudomonads. For other carbohydrates, uptake was by active transport (42, 51, 85, 88, 116, 117). As in other bacteria in which a PEP:fructose 1-phosphotransferase system operates, the sugar was accumulated as fructose-1-phosphate (107, 120, 138). Further metabolism required its conversion to fructose 1,6-diphosphate, a reaction carried out by 1-phosphofructokinase (10, 12, 107, 120, 138). This key intermediate occurs at a branch point in the dissimilation of fructose.

There are two physiologically significant paths of conversion of fructose 1,6-diphosphate to pyruvate: a minor branch forming triose phosphate via the Embden-Meyerhof reactions, and a major branch involving the cleavage of phosphate from fructose 1,6-diphosphate to form fructose-6-phosphate with subsequent metabolism of this compound via the Entner-Doudoroff pathway (9, 107, 120, 138). An unusual feature of this pathway is the atypical role of fructose 1,6-diphosphatase as a catabolic enzyme.

Both PEP:fructose phosphotransferase and 1-phosphofructokinase were induced in the presence of fructose (11, 107, 120, 138). *P. aeruginosa* mutants lacking activity of PEP:fructose phosphotransferase activity were isolated on the basis of their inability to utilize fructose (106, 114). Such mutants grew normally on mannitol, consistent with the interpretation that fructose provided in the medium was metabolized via fructose-1-phosphate, whereas fructose generated endogenously from oxidation of mannitol was metabolized via fructose-6-phosphate. A similar derivative of *P. doudoroffii* was isolated by Baumann & Baumann (11). Durham & Phibbs characterized the PEP:fructose phosphotransferase system of *P. aeruginosa* and found it was atypical in lacking an HPr-like low-molecular-weight phosphate-carrier protein (41). The *P. aeruginosa* system appears to be comprised of only two components: a soluble enzyme I of 72,000 M_r and a membrane-associated enzyme II complex.

Van Dijken & Quayle isolated *P. extorquens* mutants lacking 1-phosphofructokinase activity by selection for inability of the bacteria to utilize fructose (138). Fructose-positive revertants regained normal levels of 1-phosphofructokinase activity. This enzyme has been characterized in several pseudomonads (10, 12, 138).

The relative importance of the two routes of fructose 1,6-diphosphate utilization has been examined in *P. aeruginosa* (107; P. V. Phibbs, Jr., unpublished data). Mutants blocked in the major route of fructose utilization at the level of glucose-6-phosphate dehydrogenase (Zwf^-) were used to isolate mutants blocked in the minor route. Two classes of such mutants were obtained: Zwf^- Pyk^- strains, lacking pyruvate kinase activity, and Zwf^- Eno^- strains, lacking enolase activity. The original Zwf^- mutants grew at approximately one fifth the wild-type rate on fructose. The double mutants failed to grow on fructose. Zwf^+ revertants of these strains grew at a normal rate on fructose, indicating that the major route of fructose 1,6-diphosphate dissimilation was via the Entner-Doudoroff rather than the Embden-Meyerhof pathway. This was also confirmed by the observation that Pgi^-, Edd^-, and Eda^- mutants grew only slowly on fructose (18, 107, 115).

To fully define this pathway it will be necessary to isolate fructose 1,6-diphosphatase–deficient strains and confirm the unorthodox role of this enzyme as a major component of this and other pathways of carbohydrate dissimilation in pseudomonads. To our knowledge, *Pseudomonas* mutants deficient in fructose 1,6-diphosphatase have not been isolated. However, the properties of this enzyme have been examined in a variety of pseudomonads, where it appears to be formed constitutively (5, 9, 47, 82, 135, 138).

Fructose dissimilation in *P. cepacia* (2) and probably in certain strains of *P. saccharophila* (98) differed from that in other pseudomonads. *P. cepacia* accumulated fructose by active transport (2). Further metabolism of fructose depended upon formation of an inducible fructokinase and followed essentially

the same pathway as that for dissimilation of mannitol and sorbitol. Presumably the option for use of the Embden-Meyerhof pathway was precluded because fructose 1,6-diphosphate was not formed. Fructose was metabolized exclusively via the Entner-Doudoroff pathway. Allenza et al obtained two classes of mutants that had lost the ability to utilize fructose (2). Representatives of one retained the capacity to utilize glucose and were blocked in transport of fructose or lacking in fructokinase activity. Members of the other class were originally selected on the basis of their failure to utilize gluconate, and were lacking in either 6-phosphogluconate dehydratase or KDPG aldolase activity (3).

Mutants Blocked in Hexitol Utilization

Ability to utilize mannitol as sole carbon source is common among pseudomonads (2, 107, 128). Some species also have the capacity to utilize sorbitol and galactitol (2, 15, 128). The degradation of mannitol has been studied in detail in *P. aeruginosa* and *P. cepacia* (2, 107). In both pseudomonads, mannitol utilization involved formation of fructose, fructose-6-phosphate, glucose-6-phosphate, and 6-phosphogluconate as intermediates and was dependent upon operation of the Entner-Doudoroff pathway (see Figure 2). Sorbitol utilization in *P. cepacia* proceeded via the same intermediates (2). Components of the mannitol utilization pathway induced by growth on mannitol included: an active transport system for mannitol, mannitol dehydrogenase, fructokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and KDPG aldolase (2, 107). Both *P. aeruginosa* and *P. cepacia* formed constitutive species of phosphoglucoisomerase (2, 107). In addition, *P. cepacia* formed a second isozyme absent from extracts of bacteria grown on citrate but present in bacteria grown on mannitol, sorbitol, fructose, or glucose (2). The presence of multiple species of phosphoglucoisomerase and of glucose-6-phosphate dehydrogenase in *P. cepacia* may account for the failure to obtain mutants blocked in mannitol utilization at these steps (2, 69).

P. cepacia also formed two species of hexitol dehydrogenase: a 65,000-M_r mannitol dehydrogenase that was equally active with D-arabitol, and a 100,000-M_r sorbitol dehydrogenase that was highly active with D-galactitol (1, 2). Both enzymes were induced during growth of the bacteria on hexitols.

An important outcome of the search for mutant derivatives of *P. aeruginosa* PAO that were blocked in mannitol utilization was the isolation of strains deficient in the key phosphorylative pathway enzyme glucose-6-phosphate dehydrogenase (8, 107). Mutants blocked in the previous step, conversion of fructose-6-phosphate to glucose-6-phosphate (mediated by phosphoglucoisomerase), were also obtained by selection for mannitol-negative derivatives (107). The rather large collection of mannitol-negative strains obtained by Phibbs and his coworkers did not include fructokinase-deficient mutants. In contrast, fructokinase-deficient strains were the most common type of mutant

blocked in utilization of mannitol by *P. cepacia* (2). The fructokinase-deficient mutants failed to utilize mannitol or sorbitol, indicating that fructose-6-phosphate was an intermediate in dissimilation of sorbitol as well as of mannitol. The first enzymatic step in dissimilation of mannitol is its oxidation by an NAD-dependent mannitol dehydrogenase to fructose. Mutants lacking activity of this enzyme have been obtained both in *P. aeruginosa* (107) and in *P. cepacia* (2). The loss of mannitol dehydrogenase in *P. cepacia* was accompanied by loss of D-arabitol dehydrogenase activity associated with this enzyme and of capacity to utilize D-arabitol (1).

The initial event in mannitol utilization was its uptake by active transport (44, 104, 107). Mutants impaired in transport of mannitol have been isolated in *P. aeruginosa* (107). However, these strains exhibited multiple loss of function, including mannitol dehydrogenase activity. As expected, Edd^- and Eda^- mutants of *P. aeruginosa* and of *P. cepacia* were unable to grow on mannitol (2, 18, 107, 115). The *P. cepacia* mutants were also blocked in utilization of sorbitol.

Eisenberg & Phibbs have demonstrated that mannitol induces the formation of a 37,000-M_r mannitol-binding protein distinct from mannitol dehydrogenase (44). The protein was released from cells by osmotic shock treatment without a comparable release of cytoplasmic enzymes. The unusually high isoelectric point of this protein (pI of 8.3) facilitated its purification by isoelectric focusing.

DISSIMILATION OF GLYCEROL

Glycerol dissimilation in pseudomonads proceeds by its conversion to the central pathway intermediate D-glyceraldehyde-3-phosphate (see Figure 2). The individual steps in *P. aeruginosa* involve uptake of glycerol, its phosphorylation to form glycerol-3-phosphate, and oxidation of the latter compound to glyceraldehyde-3-phosphate (29, 82, 106, 126, 136). Growth in the presence of either glycerol or glycerol-3-phosphate resulted in a marked increase in capacity of the bacteria to transport both glycerol and glycerol-3-phosphate, as well as induction of glycerol kinase and a membrane-associated pyridine nucleotide–independent glycerol-3-phosphate dehydrogenase. Mutants deficient in the latter enzyme were unable to utilize glycerol or glycerol-3-phosphate (82).

Glycerol and glycerol-3-phosphate negative mutants were also obtained that had lost all three of these activities. The simultaneous loss of these activities appeared to be the consequence of a single mutation, suggesting that the genes encoding proteins of the glycerol dissimilation system in *P. aeruginosa* represent a common regulon.

Aparicio et al noted that *P. putida* derivatives lacking 3-phosphoglycerate

kinase were able to grow normally on glycerol (5). This was also true of *P. aeruginosa* mutants deficient in enolase or pyruvate kinase activity (P. V. Phibbs, Jr., unpublished results); whereas *P. aeruginosa* mutants blocked in the Entner-Doudoroff pathway grew poorly on glycerol (8, 18). This suggested that glyceraldehyde-3-phosphate derived from glycerol might be converted to glucose-6-phosphate and channeled into the Entner-Doudoroff pathway. The pattern of induction of enzymes related to hexose phosphate utilization was consistent with such a model. It has been well established that growth of *P. aeruginosa* on glycerol, glycerol-3-phosphate, or glycerate results in induction of glucose-6-phosphate dehydrogenase and of enzymes of the Entner-Doudoroff pathway (54, 60, 68). This is true in several pseudomonads (3, 5, 70).

In certain pseudomonads, notably in *P. putida* (where a phosphoglycerate kinase–deficient strain grew normally on glycerol) and in *P. cepacia,* mutants blocked in the Entner-Doudoroff pathway grew at a near-normal rate on glycerol, although Entner-Doudoroff pathway enzymes were induced in the wild type during growth on glycerol (3, 5). This suggested that in these bacteria the Entner-Doudoroff and Embden-Meyerhof pathways were truly alternative routes of glycerol dissimilation.

In *P. aeruginosa* PAO, deficiency of phosphoglucoisomerase or glucose-6-phosphate dehydrogenase, like a block in the Entner-Doudoroff pathway, reduced the growth rate on glycerol to about one fifth that of the wild type (107). Double mutants lacking glucose-6-phosphate dehydrogenase and in addition enolase or pyruvate kinase activities were completely unable to utilize glycerol, confirming that there was no third pathway of glycerol dissimilation (P. V. Phibbs, Jr., unpublished data). It appears unlikely that a pathway leading from glyceraldehyde-3-phosphate through D-glyceraldehyde, methylglyoxal, and lactate to pyruvate, as proposed by Rizza & Hu for a pseudomonad of uncertain origin (111), operates in *P. aeruginosa*. Cooper attempted to confirm the existence of this pathway in *P. saccharophila* and failed to do so (28). However, he identified an enzyme that converted dihydroxyacetone phosphate to methylglyoxal and suggested that it might be a component of a glycolytic by-pass system allowing for conversion of triose phosphate to pyruvate under conditions where glyceraldehyde-3-phosphate dehydrogenase activity was restricted due to limitation of phosphate.

One observation that prompted Rizza & Hu to look for novel routes of glyceraldehyde-3-phosphate dissimilation was their belief that pseudomonads lacked glyceraldehyde-3-phosphate dehydrogenase (111). It is now clear that a variety of pseudomonads including *P. aeruginosa* (5, 18, 28, 54, 95, 135) and *P. cepacia* (T. G. Lessie, unpublished observations) possess high levels of activity of this enzyme and that some other step must limit the direct conversion of glyceraldehyde-3-phosphate to pyruvate. One candidate is pyruvate kinase–

mediated conversion of phosphoenolpyruvate to pyruvate. Utter and his coworkers have made the interesting observation that this enzyme is activated by KDPG (25, 96). This suggests that metabolism of glyceraldehyde-3-phosphate via the Embden-Meyerhof pathway might depend upon operation of the Entner-Doudoroff pathway. Clearly, further studies of mechanisms controlling formation and activities of enzymes related to glyceraldehyde-3-phosphate dissimilation, such as glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and fructose 1,6-diphosphatase is warranted.

UTILIZATION OF GALACTOSE AND RELATED CARBOHYDRATES

In their classic studies of the central routes of carbohydrate utilization in *P. saccharophila,* Doudoroff and his coworkers established that this bacterium metabolized galactose via a set of reactions (38) different from those of the well-known Leloir pathway (81). The new route, which we will term the De Ley-Doudoroff pathway (see Figure 3), was similar to the Entner-Doudoroff route in two respects: (*a*) the end products were identical, pyruvate and glyceraldehyde-3-phosphate; and (*b*) the sequence included dehydratase and

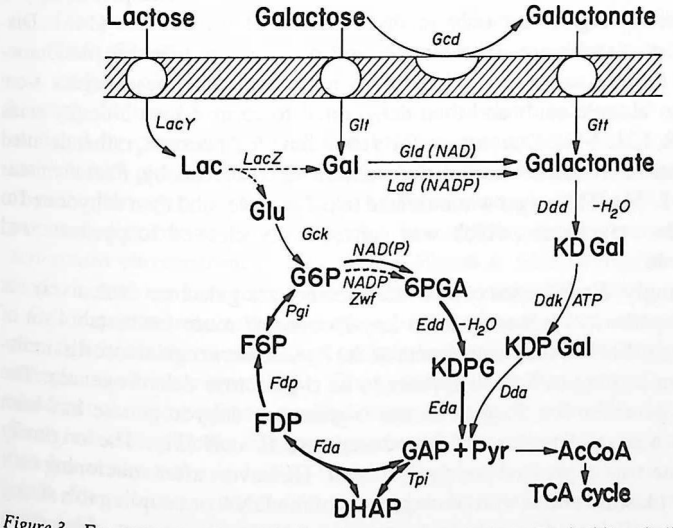


Figure 3 Enzymes related to utilization of galactose and lactose. *Dashed lines* indicate features related to *P. cepacia* strains described in the text. Abbreviations: LacY, Glt, and Gtt represent transport systems for lactose, galactose, and galactonate; LacZ, ß-galactosidase; Gld and Lad, D-galactose and L-arabinose dehydrogenases; Ddd, Ddk, and Dda represent the De Ley–Doudoroff pathway enzymes, galactonate dehydratase, 2-keto-3-deoxygalactonate kinase, and 2-keto-3-deoxy-6-phosphogalactonate aldolase. For other abbreviations see Figures 1 and 2.

aldolase reactions formally analogous to those carried out by 6-phosphogluconate dehydratase and KDPG aldolase. However, enzymes of the De Ley-Doudoroff pathway proved to be distinct from those of the Entner-Doudoroff route (124).

The first step of the De Ley-Doudoroff pathway was the oxidation of galactose to galactonate by an NAD-dependent dehydrogenase (38, 76). Galactonate was then converted to glyceraldehyde-3-phosphate and pyruvate in three steps: dehydration of galactonate to produce 2-keto-3-deoxygalactonate (38), phosphorylation of the keto-deoxy sugar by an ATP-dependent kinase producing 2-keto-3-deoxy-6-phosphogalactonate (149), and cleavage of the latter by a specific 2-keto-3-deoxy-6-phosphohexonate aldolase to form glyceraldehyde-3-phosphate and pyruvate (123, 124). It is likely that the Entner-Doudoroff pathway plays an important role in dissimilation of glyceraldehyde-3-phosphate derived by cleavage of 2-keto-3-deoxy-6-phosphogalactonate (15).

P. cepacia formed two galactose dehydrogenase species, an NAD-specific enzyme like that from *P. saccharophila,* and a membrane-associated (pyridine nucleotide–independent) dehydrogenase that proved identical to glucose dehydrogenase (15). *P. cepacia* also produced a cytoplasmic NADP-linked L-arabinose dehydrogenase that was highly active with D-galactose. However, this enzyme was not formed during growth on galactose, and presumably is physiologically significant only in dissimilation of L-arabinose (14a). Dissimilation of L-arabinose, D-arabinose, and D-fucose in different pseudomonads was similar to that of galactose to the extent that these sugars were oxidized to aldonic acids and then dehydrated to form 2-keto-3-deoxy acids (31–34, 99, 121, 143). Due to the efforts of Dahms & Anderson, rather detailed information is available on the degradation of D-fucose by *Pseudomonas* MSU-1 (31–34). This sugar was oxidized to D-fuconate, and then dehydrated to 2-keto-3-deoxyfuconate, which was subsequently cleaved to pyruvate and lactaldehyde.

Surprisingly, *Escherichia coli,* which metabolizes galactose exclusively via the Leloir pathway, possesses a De Ley-Doudoroff route for metabolism of galactonate (35). The only component of the *Pseudomonas* galactose dissimilation system lacking in *E. coli* appears to be D-galactose dehydrogenase. The structural gene for the *P. fluorescens* D-galactose dehydrogenase has been cloned on a cosmid vector and introduced into *E. coli* (21). The originally cloned gene was expressed poorly in *E. coli*. However, after subcloning on a multicopy plasmid and in vitro mutagenesis of the DNA or coupling to a strong promoter, sufficient expression of galactose dehydrogenase was obtained to support growth on D-galactose of an *E. coli* strain blocked in the Leloir pathway (21). The galactose dehydrogenases from *P. fluorescens* and *P. saccharophila* have been purified and characterized in detail (16, 144).

Pseudomonads as a group lack the capacity to utilize lactose as carbon source (100, 128). *P. maltophila* is an exception (100). Lessie and his coworkers

transferred the *lac* plasmid pGC91.14 from *E. coli* to *P. cepacia* and then selected for capacity to utilize lactose. The Lac^+ isolates formed ß-galactosidase constitutively as a consequence of insertion of *P. cepacia* DNA into the Tn*951* element of this plasmid (93). The results differed from those of Baumberg et al, who found that *lac* genes of pGC91.14 were expressed poorly in *P. aeruginosa* (13). Nano & Kaplan found the *lac* genes on this plasmid were poorly expressed in *Rhodopseudomonas spheroides* (90). The latter investigators were able to select Lac^+ derivatives with somewhat better expression. During growth of the Lac^+ isolates of *P. cepacia* on lactose, D-galactose dehydrogenase and enzymes of both the Entner-Doudoroff and De Ley-Doudoroff pathway were induced (14a). Growth on lactose also resulted in hyperinduction of enzymes of the phosphorylative pathway of glucose dissimilation as noted during growth of *P. cepacia* on the disaccharides trehalose and cellobiose (14).

GENETIC MAPPING OF CARBOHYDRATE UTILIZATION GENES

Largely through the efforts of Holloway and his coworkers, who developed the use of drug-resistance plasmids to mobilize chromosomal markers in *P. aeruginosa* PAO (52, 56, 57, 118), it has become possible to undertake a comprehensive analysis of genes related to carbohydrate utilization in this bacterium. Recently Phibbs, Matsumoto, and their coworkers have used conjugation mediated by plasmids R68.45 and FP2, in conjunction with transductional analysis with phages F116L and G101, to establish the chromosomal locations of genes that specify carbohydrate utilization enzymes (29, 113–115). The proposed distribution of these genes on an abbreviated version of the *P. aeruginosa* chromosome is indicated in Figure 4. More complete listings of chromosomal markers have been published recently (94, 118).

One important result of this work was the finding that *zwf, edd,* and *eda* loci were tightly clustered and linked to *nalA* in the 50–55 minute region of the *P. aeruginosa* chromosome (115). The possibility that these genes may be organized into the same operon merits further investigation. Another locus specific for an inducible glucose transport system, *glt,* also exhibited a high cotransduction frequency with *zwf-1* (30). The gene for glycerophosphate dehydrogenase, *glpD,* likewise was mapped in this region by demonstrating cotransduction with *nalA, zwf-1, eda-9001, edd-1,* and with *amiE* (29).

Loci affecting expression of the PEP:fructose 1-phosphotransferase system, *pts,* were situated in another region near 45 min (114). These loci were cotransducible with *argF10* and *lys-9015,* and appeared to include both regulatory and structural genes. The gene that encodes pyruvate carboxylase, *pyc,* also exhibited coinheritance with *argF10* in R68.45-mediated conjugations

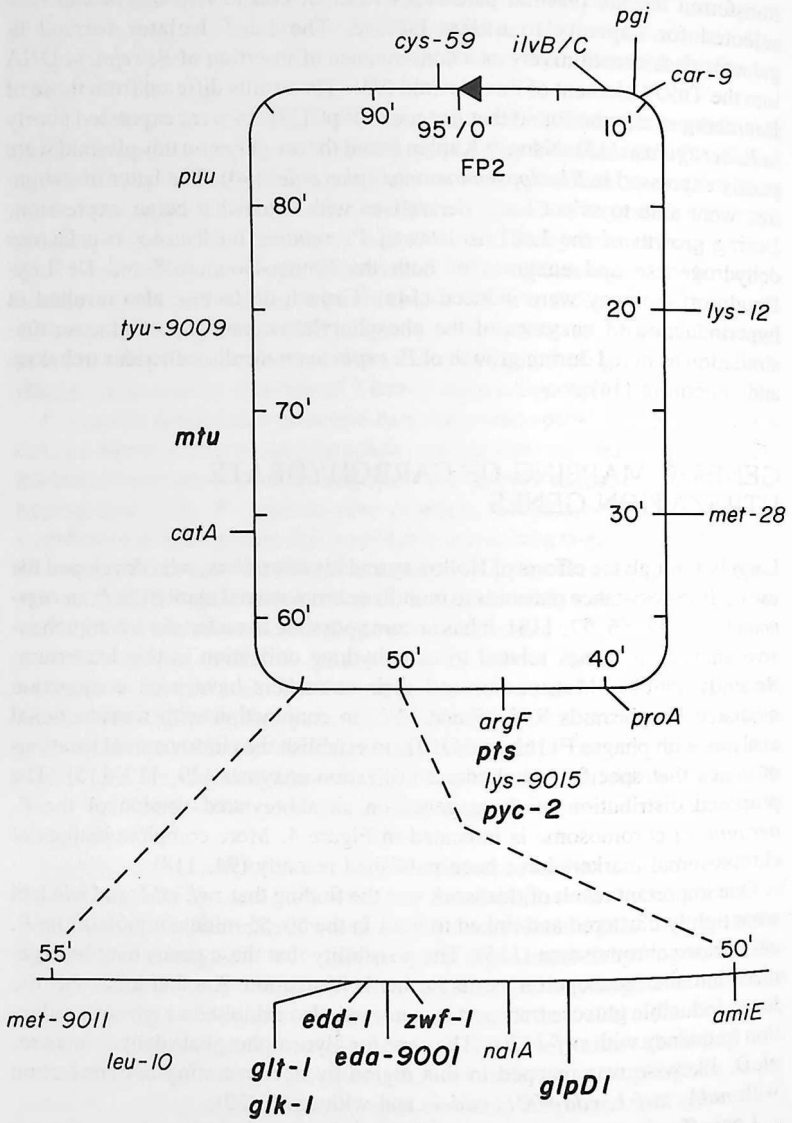


Figure 4 Distribution of genes specifying carbohydrate utilization on the chromosome of *P. aeruginosa* strain PAO1. Locations for markers not associated with carbohydrate utilization are based on a recently revised and recalibrated map (B. W. Holloway, personal communication). The proposed locations and relative ordering of markers in the expanded 50–55 minute region (not drawn to exact scale) are based on cotransductions performed with phages F116L and G101.

(115). Pyruvate carboxylase is an inducible enzyme essential for growth on glucose and other carbohydrates (103). It plays a key role in replenishment of oxaloacetate required for entry of acetyl-coenzyme A into the TCA cycle.

A set of mutations affecting mannitol utilization, *mtu*, was mapped by conjugal matings near 70 min, between *catA* and *tyu-9009* (113, 118). These mutations appeared to define a mannitol operon including structural genes for an inducible mannitol-binding protein, mannitol dehydrogenase, and 6-fructokinase. Several dozen independently isolated, spontaneously revertible *mtu* mutations were characterized. All caused a deficiency of mannitol dehydrogenase, most were deficient in one additional function, and several failed to express all components unique to the mannitol catabolic pathway. Quantitative two-factor transductions performed with phage F116L demonstrated clustering of these mutations into two regions, corresponding to a putative regulatory locus, *mtuR*, and to the structural gene for mannitol dehydrogenase, *mtuD*.

The gene that encodes phosphoglucoisomerase, *pgi*, an enzyme essential for mannitol utilization, recently was mapped in the early region of the *P. aeruginosa* chromosome. Three-factor transductional and conjugal crosses located *pgi* at about 8 min, between *ilvB/C* and *car-9* (P. V. Phibbs, Jr., H. Matsumoto & J. Calligeros; unpublished results).

Recent maps of the *P. aeruginosa* chromosome (94, 115, 118) indicate that the majority of carbohydrate utilization genes are situated in the 45–70-min. region of the chromosome. A similar nonrandom distribution of carbohydrate utilization genes has been reported in *P. putida* (36, 40). At present the reason for clustering of carbohydrate utilization genes in a particular region of the chromosome is unknown. It will be interesting to see whether such clustering of carbohydrate utilization genes extends to other pseudomonads. One promising approach developed by Holloway and his coworkers that should be useful for such analysis is complementation mapping (87). In this procedure R' derivatives are generated from broad host range drug-resistance plasmids in one species or genus and then screened for capacity to complement well characterized mutations in another species or genus.

It is anticipated that cloning of *Pseudomonas* carbohydrate utilization genes will prove a useful tool in future efforts to gain information about the organization and regulation of their expression. We have already discussed the case of

Fig. 4 (cont.) Markers for inducible carbohydrate catabolic functions are illustrated by heavy lettering. Symbols: *ami*, amidase (acetamide utilization); *car*, carbamoyl phosphate synthetase; *cat*, catechol utilization; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *edd*, 6-phosphogluconate dehydratase; *glk*, glucokinase; *glt*, glucose transport system; *glpD*, membrane-associated glycerol-3-phosphate dehydrogenase; *mtu*, mannitol utilization; *nal*, nalidixic acid resistance; *pgi*, phosphoglucoisomerase; *pts*, PEP:fructose 1-phosphotransferase system; *puu*, purine utilization, *pyc*, pyruvate carboxylase; *tyu*, tyrosine utilization; *zwf*, glucose-6-phosphate dehydrogenase.

the galactose dehydrogenase gene from *P. fluorescens*, which was cloned on an *E. coli* cosmid vector (21). Nester and his coworkers have developed a similar cosmid vector from the wide host range plasmid RP1, which should facilitate cloning of *Pseudomonas* genes and examination of factors that regulate their expression (63). Several multicopy plasmid vectors have been developed that are suitable for complementation studies in *Pseudomonas* species (6, 97, 119, 150).

For example, Phibbs and his collaborators recently used a wide host range plasmid, pR01769, developed by R. H. Olsen, to clone an ~ 4-Mdal *Eco*RI fragment of the *P. aeruginosa* chromosome (30). This fragment appeared to carry structural genes for 6-phosphogluconate dehydratase, glucokinase, one or more proteins involved in glucose transport, and a regulatory locus affecting *zwf, edd, eda,* and *glk* expression. These results confirmed the map location of the *glt* locus determined earlier by transductional analysis, and provided the first evidence that *glk,* the gene that encodes glucokinase, is also part of the same cluster that contains *edd, eda,* and *zwf* genes.

REPRESSION AND INHIBITION OF DEGRADATIVE ENZYMES

Pseudomonads grow rapidly with tricarboxylic acid (TCA) cycle intermediates such as citrate, succinate, and malate as sole source of carbon and energy. When the bacteria are presented with a mixture of carbohydrates and TCA cycle compounds, diauxic growth ensues, and the TCA cycle compounds are utilized before the carbohydrates (4, 18, 60, 73, 135). Such diauxie is related to repression of carbohydrate utilization enzymes and transport systems (60, 73, 85, 92, 148). However, the control mechanisms that preclude the formation of affected carbohydrate utilization enzymes and assure preferential utilization of TCA cycle intermediates remain to be defined.

Dawes and his coworkers compared the influence of citrate on chemostat cultures of *P. aeruginosa* limited by the supply of ammonium ion, and noted a marked decrease in capacity of the bacteria to transport glucose and form direct oxidative, phosphorylative, and Entner-Doudoroff pathway enzymes as the concentration of citrate was increased relative to that of glucose (92, 147). Lynch & Franklin found that malate blocked uptake of gluconate and 2-ketogluconate by *P. fluorescens,* but did not affect conversion of glucose to 2-ketogluconate (73). The results suggested that, at least in this pseudomonad, enzymes of the direct oxidative pathway leading from glucose to 2-ketogluconate were resistant to catabolite repression.

Siegel et al examined the cyclic AMP content of *P. aeruginosa* on glucose in the presence and absence of TCA cycle intermediates (repressive and non-repressive conditions) and failed to find significant differences in intracellular

levels of this compound (125). Furthermore, addition of exogenous cAMP failed to reverse succinate-mediated repression of glucose-6-phosphate dehydrogenase or of mannitol dehydrogenase. These results in conjunction with the studies of repression of histidase in *P. putida* and *P. aeruginosa* by Phillips & Mulfinger (108) suggest that cyclic AMP–mediated control is not a component of catabolite repression in pseudomonads as it is in other bacteria (19).

Dawes and his coworkers have examined in detail the interplay between the direct oxidative and phosphorylative pathway in *P. aeruginosa* and concluded that gluconate plays a critical role in governing the operation of the phosphorylative pathway (85, 147, 148). Gluconate was shown both to inhibit the transport of glucose into the cell and to repress formation of the glucose-transport system. At sufficiently high levels of glucose, gluconate formed by the action of glucose dehydrogenase repressed the glucose-transport system (but not the transport system for gluconate and 2-ketogluconate) and assured preferential dissimilation of glucose via the direct oxidative pathway (148). Glucose dehydrogenase–deficient mutants were uncoupled with respect to such control, presumably due to their failure to convert glucose to gluconate (85, 147). The hyperinduction of phosphorylative pathway enzymes and of glucose transport capacity during growth of glucose dehydrogenase–deficient mutants of *P. cepacia* on glucose or of wild-type *P. cepacia* on cellobiose or trehalose may be in part a manifestation of the same phenomenon (14).

The key phosphorylative pathway enzyme glucose-6-phosphate dehydrogenase is subject to strong inhibition by ATP (17, 68–70, 80, 122, 137), which decreases its affinity for NAD and/or D-glucose-6-phosphate. Two species of this enzyme were detected in *P. cepacia:* a 240,000-M_r, ATP-sensitive enzyme active with NAD or NADP and a 120,000-M_r NADP-specific enzyme (22, 69, 137). Both were comprised of subunits of about 60,000 daltons and tended to aggregate in the presence of D-glucose-6-phosphate. However, the two enzymes were immunologically distinct, indicating they did not represent different oligomeric forms of the same gene product (22, 137). *P. fluorescens* also formed two species of glucose-6-phosphate dehydrogenase: a major ATP-sensitive enzyme and a minor ATP-insensitive isozyme (70). *Pseudomonas aeruginosa* contained only one glucose-6-phosphate dehydrogenase, which was strongly inhibited by ATP (68).

It has been suggested that inhibition of glucose-6-phosphate dehydrogenase by ATP serves to prevent wasteful degradation of ATP under conditions of gluconeogenesis, where the hexose phosphate formed is essential for biosynthesis of cell wall material and other macromolecules precursors (68). It is possible that under certain conditions the same mechanism of ATP-dependent control of glucose-6-phosphate dehydrogenase might play a role in regulation of the formation of certain carbohydrate utilization enzymes, for example, in *P. aeruginosa* and *P. cepacia,* where 6-phosphogluconate appears

to be the physiological inducer of the Entner-Doudoroff pathway enzymes (3, 107). ATP-linked inhibition of glucose-6-phosphate dehydrogenase might block induction of Entner-Doudoroff pathway enzymes by reducing 6-phosphogluconate levels below the threshold required for induction.

CONCLUSIONS

The pioneer studies of Doudoroff, Wood, and others of glucose dissimilation in pseudomonads demonstrated that conversion of glucose to glyceraldehyde-3-phosphate and pyruvate proceeded by cleavage of KDPG, a metabolite unique to the Entner-Doudoroff pathway (37, 45, 64, 65). 6-Phosphogluconate, the immediate precursor of KDPG, was recognized as a key intermediate of carbohydrate dissimilation and a potential source of pentose-phosphate via the hexose monophosphate pathway. Fructose 1,6-diphosphate was not considered to play a significant role in dissimilation of carbohydrates, because the absence of 6-phosphofructokinase (54, 95, 120, 135) appeared to preclude its formation from glucose or other carbohydrates. Glyceraldehyde-3-phosphate, derived from hexoses or glycerol, was presumed to be metabolized to pyruvate exclusively through the lower portion of the Embden-Meyerhof route and ultimately oxidized via the tricarboxylic acid cycle.

The recent discovery of a PEP:fructose phosphotransferase system in pseudomonads (11, 41, 114, 120, 138) and the analysis of carbohydrate-negative mutants have provided a different perspective of the roles of fructose 1,6-diphosphate. It now appears that this compound is a key intermediate in the catabolism of fructose and more importantly in the metabolism of glyceraldehyde-3-phosphate formed from other carbohydrates. Appreciation of this role of fructose 1,6-diphosphate has led to the recognition that fructose 1,6-diphosphatase functions as a catabolic enzyme in addition to its more orthodox activity in gluconeogenesis. The proposed role of fructose-1,6-diphosphatase as a component of the pathway leading from glyceraldehyde-3-phosphate to KDPG needs to be confirmed by mutant studies. To our knowledge mutants lacking this enzyme have not been isolated. Efforts also need to be made to identify factors that limit the direct conversion of glyceraldehyde-3-phosphate to pyruvate and favor its recycling into the Entner-Doudoroff pathway.

Progress has been made in identification of regulatory phenomena affecting carbohydrate utilization in pseudomonads. However, this area needs to be explored further. For example, the actual mechanisms governing the strong repression of carbohydrate-utilization enzymes and transport systems by tricarboxylic acid cycle intermediates remain to be established. It should be emphasized that the type of cyclic-AMP related control that underlies catabolite repression in members of the *Enterobacteriaceae* appears not to operate in two pseudomonads (108, 125), pointing to the possibility that other major mechanisms of control of catabolic pathways remain to be discovered in these bacteria. The isolation of mutants uncoupled with respect to ATP-linked control of glucose-6-phosphate dehydrogenase would represent an important contribution in sorting out another aspect of regulation of carbohydrate utilization. To our knowledge there have been no reports describing the consequences of loss of nucleotide-dependent control of any catabolic enzyme.

The refinement over the past few years of systems of genetic analysis in *P. aeruginosa* PAO has afforded new opportunities to examine control of gene expression in this bacterium. Systems of gene exchange have also been reported for other pseudomonads (24, 52, 56, 57), and we anticipate that similar systems will be developed in the near future for most pseudomonads. Thus we are likely to see over the next few years considerable progress being made towards understanding genetic mechanisms underlying the extraordinary catabolic potential of *Pseudomonas* species.

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Literature Cited

1. Allenza, P. 1983. *PhD Thesis, University of Massachusetts/Amherst* 95 pp.
2. Allenza, P., Lee, Y. N., Lessie, T. G. 1982. *J. Bacteriol.* 150:1348–56
3. Allenza, P., Lessie, T. G. 1982. *J. Bacteriol.* 150:1340–47
4. Anderson, R. L., Wood, W. R. 1969. *Ann. Rev. Microbiol.* 23:539–78
5. Aparicio, M., Ruiz-Amil, M., Vicente, M., Canovas, J. L. 1971. *FEBS Lett.* 14:326–28
6. Bagdasarian, M., Timmis, K. N. 1982. *Curr. Top. Microbiol. Immunol.* 96:48–67
7. Ballard, R. W., Palleroni, N. J., Doudoroff, M., Stanier, R. Y., Mandel, M. 1970. *J. Gen. Microbiol.* 60:199–214
8. Banerjee, P. C., Vanags, R. I., Chakrabarty, A. M., Maitra, P. K. 1983. *J. Bacteriol.* 155:238–45
9. Bang, S. S., Baumann, P. 1978. *Curr. Microbiol.* 1:5–9
10. Bang, S. S., Baumann, P., Sawyer, M. H. 1977. *Can. J. Microbiol.* 23:721–25
11. Baumann, P., Baumann, L. 1975. *Arch. Microbiol.* 105:225–40
12. Baumann, L., Baumann, P. 1975. *Arch. Microbiol.* 105:241–48
13. Baumberg, S., Cornelis, G., Panagiotakopoulos, M., Roberts, M. 1980. *J. Gen. Microbiol.* 119:257–62
14. Berka, T., Allenza, P., Lessie, T. G. 1984. *Curr. Microbiol.* In press
14a. Berka, T. 1984. *PhD thesis,* Univ. Mass./Amherst. 145 pp.
15. Berka, T., Lessie, T. G. 1984. *Curr. Microbiol.* In press
16. Blachnitzky, E-O., Wengenmayer, F., Kurz, G. 1974. *Eur. J. Biochem.* 47:235–50
17. Blackkolb, F., Schlegel, H. G. 1968. *Arch. Mikrobiol.* 63:177–96
18. Blevins, W. T., Feary, T. W., Phibbs, P. V. Jr. 1975. *J. Bacteriol.* 121:942–49
19. Botsford, J. L. 1981. *Microbiol. Rev.* 45:620–42
20. Bowien, B., Schlegel, H. G. 1972. *Arch. Mikrobiol.* 87:221–34
21. Buckel, P., Zehelein, E. 1981. *Gene* 16:149–59
22. Cacciapuoti, R. F., Lessie, T. G. 1977. *J. Bacteriol.* 132:555–63
23. Campbell, J. J. R., Hogg, L. A., Strasdine, G. A. 1962. *J. Bacteriol.* 83:1155–60
24. Carlson, C. A., Pierson, L. S., Rosen, J.

J., Ingraham, J. L. 1983. *J. Bacteriol.* 153:93–99
25. Chuang, D. T., Utter, M. F. 1979. *J. Biol. Chem.* 254:8434–41
26. Coffee, C. J., Hu, A. S. L. 1970. *J. Biol. Chem.* 245:3703–07
27. Coffee, C. J., Hu, A. S. L. 1972. *Arch. Biochem. Biophys.* 149:549–59
28. Cooper, R. A. 1974. *Eur. J. Biochem.* 44:81–86
29. Cuskey, S. M., Phibbs, P. V. Jr. 1983. *Abstr. Ann. Meet. Amer. Soc. Microbiol., New Orleans* K39, p. 183
30. Cuskey, S. M., Wolff, J. A., Olsen, R. H., Phibbs, P. V. Jr. 1984. *Abstr. Ann. Meet. Amer. Soc. Microbiol., St. Louis* In press
31. Dahms, A. S., Anderson, R. L. 1972. *J. Biol. Chem.* 247:2222–27
32. Dahms, A. S., Anderson, R. L. 1972. *J. Biol. Chem.* 247:2228–32
33. Dahms, A. S., Anderson, R. L. 1972. *J. Biol. Chem.* 247:2233–37
34. Dahms, A. S., Anderson, R. L. 1972. *J. Biol. Chem.* 247:2238–41
35. Deacon, J., Cooper, R. A. 1977. *FEBS Lett.* 77:201–5
36. Dean, H. F., Morgan, A. F. 1983. *In Genetic Maps*, ed. S. J. O'Brien, 2:128–29. Cold Spr. Harb. Lab.
37. De Ley, J. 1960. *J. Appl. Bacteriol.* 23:400–41
38. De Ley, J., Doudoroff, M. 1957. *J. Biol. Chem.* 227:745–57
39. De Torrontegui, G., Diaz, R., Canovas, J. L. 1976. *Arch. Microbiol.* 110:43–48
40. De Torrontegui, G., Diaz, R., Wheelis, M. L., Canovas, J. L. 1976. *Molec. Gen. Genet.* 144:307–11
41. Durham, D. R., Phibbs, P. V. Jr. 1982. *J. Bacteriol.* 149:534–41
42. Eisenberg, R. C., Butters, S. J., Quay, S. C., Friedman, S. B. 1974. *J. Bacteriol.* 120:147–53
43. Eisenberg, R. C., Dobrogosz, W. J. 1967. *J. Bacteriol.* 93:941–49
44. Eisenberg, R. C., Phibbs, P. V. Jr. 1982. *Curr. Microbiol.* 7:229–34
45. Entner, N., Doudoroff, M. 1952. *J. Biol. Chem.* 196:853–62
46. Feary, T. W., Blevins, W. T., Phibbs, P. V. Jr. 1975. *Abstr. Ann. Meet. Amer. Soc. Microbiol.* K34, p. 152
47. Fossit, D. D., Bernstein, I. A. 1963. *J. Bacteriol.* 86:598–99
48. Fossit, D. D., Bernstein, I. A. 1963. *J. Bacteriol.* 86:1326–31
49. Fraenkel, D. G., Vinopal, R. T. 1973. *Ann. Rev. Microbiol.* 27:69–100
50. Frampton, E. W., Wood, W. A. 1961. *J. Biol. Chem.* 236:2571–77
51. Guymon, L. F., Eagon, R. G. 1974. *J. Bacteriol.* 117:1261–69
52. Haas, D., Holloway, B. W. 1978. *Mol. Gen. Genet.* 158:229–37
53. Hauge, J. G. 1970. *Methods in Enzymol.* 9:92–98
54. Heath, H. E., Gaudy, E. T. 1978. *J. Bacteriol.* 136:638–46
55. Hochster, R. M., Katznelson, H. 1958. *Can. J. Biochem. Physiol.* 36:669–89
56. Holloway, B. W. 1979. *Plasmid.* 2:1–19
57. Holloway, B. W., Krishnapillai, Morgan, A. F. 1979. *Microbiol. Rev.* 43:73–102
58. Hunt, J. C., Phibbs, P. V. Jr. 1981. *Biochem. Biophys. Res. Commun.* 102:1393–99
59. Hunt, J. C., Phibbs, P. V. Jr. 1983. *J. Bacteriol.* 154:793–802
60. Hylemon, P. B., Phibbs, P. V. Jr. 1972. *Biochem. Biophys. Res. Commun.* 48:1041–48
61. Kersters, K., De Ley, J. 1968. *Antonie van Leewenhoek* 34:388–92
62. Kersters, K., De Ley, J. 1968. *Antonie van Leewenhoek* 34:393–408
63. Knauf, V. C., Nester, E. W. 1982. *Plasmid* 8:45–54
64. Kovachevich, R., Wood, W. A. 1955. *J. Biol. Chem.* 213:745–56
65. Kovachevich, R., Wood, W. R. 1955. *J. Biol. Chem.* 213:757–67
66. Lee, Y. N., Lessie, T. G. 1974. *J. Bacteriol.* 120:1043–57
67. Lessie, T. G., Berka, T., Zamanigian, S. 1979. *J. Bacteriol.* 139:323–25
68. Lessie, T. G., Neidhardt, F. C. 1967. *J. Bacteriol.* 93:1337–45
69. Lessie, T. G., Vander Wyk, J. C. 1972. *J. Bacteriol.* 110:1107–17
70. Lessmann, D., Schimz, K.-L., Kurz, G. 1975. *Eur. J. Biochem.* 59:545–59
71. Lockwood, L. B., Tabenkin, B., Ward, G. E. 1941. *J. Bacteriol.* 42:51–61
72. Lynch, W. H., Franklin, M. 1978. *Canad. J. Microbiol.* 24:56–62
73. Lynch, W. H., Franklin, M. 1978. *Arch. Microbiol.* 118:133–40
74. Lynch, W. H., MacLeod, J., Franklin, M. 1975. *Can. J. Microbiol.* 21:1553–59
75. Lynch, W. H., MacLeod, J., Franklin, M. 1975. *Can. J. Microbiol.* 21:1560–72
76. Maier, E., Kurz, G. 1982. *Methods Enzymol.* 89:176–81
77. Matsushita, K., Ameyama, M. 1982. *Methods Enzymol.* 89:149–54
78. Matsushita, K., Shinagawa, E., Adachi, O., Ameyama, M. 1979. *J. Biochem.* 86:249–56
79. Matsushita, K., Shinagawa, E., Ameyama, M. 1982. *Methods Enzymol.* 89:187–93
80. Maurer, P., Lessmann, D., Kurz, G. 1982. *Methods Enzymol.* 89:261–70
81. Maxwell, E. S., Kurahashi, K., Kalckar, H. M. 1962. *Methods Enzymol.* 5:174–89
82. McCowen, S. M., Phibbs, P. V. Jr., Feary, T. W. 1981. *Curr. Microbiol.* 5:191–96
83. Meloche, H. P., Ingram, J. M., Wood, W. A. 1966. *Methods in Enzymol.* 9:520–24
84. Meloche, H. P., Wood, W. A. 1966. *Methods in Enzymol.* 9:653–56
85. Midgley, M., Dawes, E. A. 1973. *Biochem. J.* 132:141–54
86. Mitchell, C. G., Dawes, E. A. 1982. *J. Gen. Microbiol.* 128:49–59
87. Moore, A. T., Nayudu, M., Holloway, B. M. 1983. *J. Gen. Microbiol.* 129: 785–99
88. Mukadda, A. J., Long, G. L., Romano, R. H. 1973. *Biochem. J.* 132:155–62
89. Nandadasa, H. G., Andreesen, M., Schlegel, H. G. 1974. *Arch. Microbiol.* 99:15–23
90. Nano, F., Kaplan, S. 1982. *J. Bacteriol.* 152:924–27
91. Narrod, S. A., Wood, W. A. 1956. *J. Biol. Chem.* 220:45–55
92. Ng, F. M. W., Dawes, E. A. 1973. *Biochem. J.* 132:129–40
93. Norskey, G., Gaffney, T., Lessie, T. G. 1984. *Abstr. Ann. Meet. Amer. Soc. Microbiol., St. Louis,* H42:98
94. O'Brien, S. J. 1983. *See Ref. 36.* pp. 165–67
95. O'Brien, R. W. 1975. *Arch. Microbiol.* 103:71–76
96. O'Brien, R., Chuang, D. T., Taylor, B. L., Utter, M. F. 1977. *J. Biol. Chem.* 252:1257–63
97. Olsen, R. H., DeBusscher, G. McCombie, W. R. 1982. *J. Bacteriol.* 150:60–69
98. Palleroni, N. J., Contopoulou, R., Doudoroff, M. 1956. *J. Bacteriol.* 71:202–7
99. Palleroni, N. J., Doudoroff, M. 1957. *J. Bacteriol.* 74:180–85
100. Palleroni, N. J., Doudoroff, M. 1972. *Ann. Rev. Phytopathol.* 10:73–100
101. Palumbo, S. A., Witter, L. D. 1969. *Appl. Microbiol.* 18:137–41
102. Palumbo, S. A., Witter, L. D. 1969. *Canad. J. Microbiol.* 15:995–1000
103. Phibbs, P. V. Jr., Feary, T. W., Blevins, W. T. 1974. *J. Bacteriol.* 118:999–1009
104. Phibbs, P. V. Jr., Eagon, R. G. 1970. *Arch. Biochem. Biophys.* 138:470–82
105. Phibbs, P. V. Jr., McNamee, C. 1976. *Abstr. Ann. Meet. Am. Soc. Microbiol.* K184, p. 167
106. Phibbs, P. V. Jr., McCowen, S. M., Feary, T. W. 1979. *Abstr. Ann. Meet. Am. Soc. Microbiol.* K122, p. 165
107. Phibbs, P. V. Jr., McCowen, S. M., Feary, T. W., Blevins, W. T. 1978. *J. Bacteriol.* 133:717–28
108. Phillips, A. T., Mulfinger, L. M. 1981. *J. Bacteriol.* 145:1286–92
109. Quay, S. C., Friedman, S. B., Eisenberg, R. C. 1972. *J. Bacteriol.* 112:291–98
110. Reber, H. H., Kaiser, P. 1981. *Arch. Microbiol.* 130:243–47
111. Rizza, Y., Hu, A. S. L. 1973. *Biochem. Biophys. Res. Commun.* 54:168–75
112. Roberts, B. K., Midgley, M., Dawes, E. A. 1973. *J. Gen. Microbiol.* 78:319–29
113. Roehl, R. A., Phibbs, P. V. Jr. 1981. *Abstr. Ann. Meet. Am. Soc. Microbiol.* K70, p. 149
114. Roehl, R. A., Phibbs, P. V. Jr. 1982. *J. Bacteriol.* 149:897–905
115. Roehl, R. A., Feary, T. W., Phibbs, P. V. Jr. 1983. *J. Bacteriol.* 156:1123–29
116. Romano, A. H., Eberhard, S. J., Dingle, S. L., McDowell, T. D. 1970. *J. Bacteriol.* 104:808–13
117. Romano, A. H., Voytek, A., Bruskin, A. M. 1980. *J. Bacteriol.* 142:755–62
118. Royle, P. L., Matsumoto, H., Holloway, B. L. 1981. *J. Bacteriol.* 145:145–55
119. Sakaguchi, K. 1982. *Curr. Top. Microbiol. Immunol.* 96:31–46
120. Sawyer, M. H., Baumann, P., Baumann, L., Berman, S. M., Canovas, J. L., et al. 1977. *Arch. Microbiol.* 112:49–55
121. Schimz, K.-L., Kurz, G. 1973. *Hoppe-Seyler's Z. Physiol. Chem.* 112:169–72
122. Schindler, J., Schlegel, H. G. 1969. *Arch. Mikrobiol.* 66:69–78
123. Shuster, C. W. 1966. *Methods in Enzymol.* 9:524–28
124. Shuster, C. W., Doudoroff, M. 1967. *Arch. Mikrobiol.* 59:279–86
125. Siegel, L. S., Hylemon, P. B., Phibbs, P. V. Jr. 1977. *J. Bacteriol.* 129:87–96
126. Siegel, L. S., Phibbs, P. V. Jr. 1979. *Curr. Microbiol.* 2:251–56
127. Spangler, W. J., Gilmour, C. M. 1966. *J. Bacteriol.* 91:245–50
128. Stanier, R. Y., Palleroni, N. J., Doudoroff, M. 1966. *J. Gen. Microbiol.* 43: 159–271
129. Stern, I. J., Wang, C. H., Gilmour, C. M. 1960. *J. Bacteriol.* 79:601–11
130. Stinnett, J. D., Guymon, L. F., Eagon, R. G. 1973. *Biochem. Biophys. Res. Commun.* 52:284–90
131. Stinson, M. W., Cohen, M. R., Merrick, J. M. 1976. *J. Bacteriol.* 128:573–79
132. Stinson, M. W., Cohen, M. A., Merrick, J. M. 1977. *J. Bacteriol.* 131:672–81
133. Stokes, F. N., Campbell, J. J. R. 1951. *Arch. Biochem. Biophys.* 30:121–25
134. Stournaras, C., Maurer, P., Kurz, G. 1983. *Eur. J. Biochem.* 130:391–96
135. Tiwari, N. P., Campbell, J. R. 1969. *Biochim. Biophys. Acta* 192:395–401

136. Tsay, S. S., Brown, K. K., Gaudy, E. T. 1971. *J. Bacteriol.* 108:82–88
137. Vander Wyk, J. C., Lessie, T. G. 1974. *J. Bacteriol.* 120:1033–42
138. Van Dijken, J. P., Quayle, J. R. 1977. *Arch. Microbiol.* 114:281–86
139. Vicente, M., Canovas, J. L. 1973. *J. Bacteriol.* 116:908–14
140. Vicente, M., Canovas, J. L. 1973. *Arch. Mikrobiol.* 93:53–64
141. Vicente, M., De Pedro, M. A., De Torrontegui, G., Canovas, J. L. 1975. *J. Mol. Cell. Biochem.* 7:59–64
142. Vicente, M., De Pedro, M. A., De Torrontegui, G., Canovas, J. L. 1975. *Arch. Microbiol.* 102:163–66
143. Weimberg, R., Doudoroff, M. 1955. *J. Biol. Chem.* 217:607–24
144. Wengenmayer, F., Ueberschar, K-H., Kurz, G. 1973. *Eur. J. Biochem.* 40:49–61
145. Wettermark, M. H., Taylor, J. R., Rogers, M. L., Heath, E. E. 1979. *J. Bacteriol.* 138:418–24
146. Whitfield, C., Sutherland, I. W., Cripps, R. E. 1982. *J. Gen. Microbiol.* 128:981–85
147. Whiting, P. H., Midgley, M., Dawes, E. A. 1976. *J. Gen. Microbiol.* 92:304–10
148. Whiting, P. H., Midgley, M., Dawes, E. A. 1976. *Biochem. J.* 154:659–68
149. Wilkinson, J. F., Doudoroff, M. 1964. *Science* 144:569
150. Wood, D. O., Hollinger, M. F., Tindol, M. B. 1981. *J. Bacteriol.* 145:1448–51
151. Wood, W. A., Schwerdt, R. F. 1957. *J. Biol. Chem.* 206:625–35

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MONOCLONAL ANTIBODIES: A Powerful Tool for Selecting and Analyzing Mutations in Antigens and Antibodies

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INTRODUCTION

Antibodies have played an important role in microbiology. In addition to their uses in classifying microorganisms and studying evolutionary relationships, antibodies are useful for detecting the microheterogeneity of proteins that arises from recombination and somatic mutation. However, the usefulness of immunological assays has been complicated by the difficulty of repeatedly generating large amounts of antibodies with restricted reactivity, and the heterogeneity of all antisera obtained by conventional methods. These problems reflect the fact that antisera contain antibodies secreted by many antibody-producing B cell clones; hence such antibodies are termed polyclonal antibodies. Even if the same antigen preparation and inbred animals are used, B cells recognizing different antigenic determinants are activated in each animal so that each antiserum contains a different spectrum of antibodies. Furthermore, the spectrum of antibodies changes during the course of an immune response, so that an animal initially making useful antibodies may subsequently make less useful ones; the reverse situation may also occur.

All investigators using conventional antisera face the problem of first obtaining the desired antibodies and then of having to generate an antiserum with similar properties once a useful antiserum is depleted. A more fundamental problem arises because the polyclonality of antisera results in multiple antibodies against different parts of each antigen as well as antibodies against many different antigens. If the antigenic determinant recognized by one of these many antibodies is lost, its loss is likely to go undetected because the vast majority of antibodies will still bind the antigen.

The usefulness of antibodies as research tools was greatly facilitated by the discovery by Kohler & Milstein of a technique for routinely producing monoclonal antibodies (59). A monoclonal antibody is secreted by the clonal progeny of a single B lymphocyte that has been immortalized by fusing it to cells from a malignant plasma cell (myeloma) line. Such immortalized B cells, called hybridomas, inherit their immortality from the myeloma parent and the genes encoding a particular antibody from the normal B cell. Hybridomas can be frozen and recovered or injected into mice to produce ascites fluid containing large amounts of the monoclonal antibody. These properties enable many different investigators to use the same antibody and allow the supply of that antibody to be renewed indefinitely.

Production of Monoclonal Antibodies

Figure 1 outlines the basic procedure for making hybridomas. Many variations on the basic technology (36, 39, 49, 63) have been published. Currently we use the one described by Fazekas de St. Groth & Scheidegger (36). Mice or rats are immunized until they make a good serum response. Once the amount of

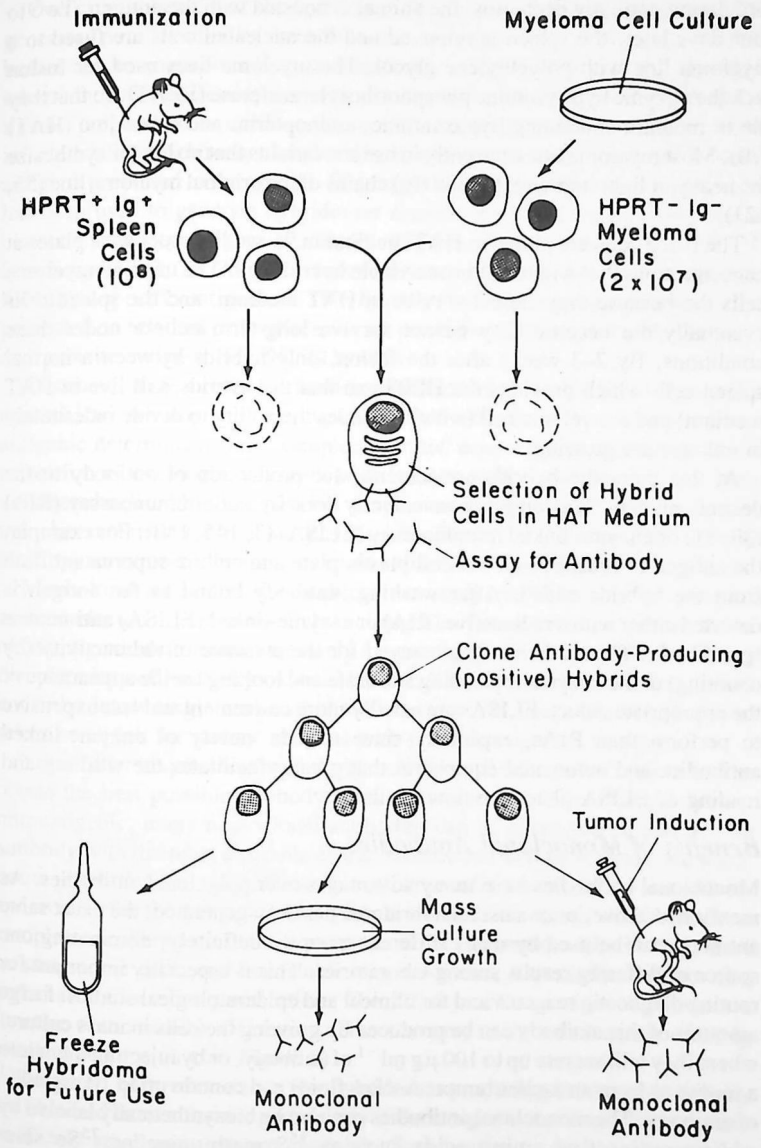


Figure 1 Production of monoclonal antibodies. Reproduced from *The New England Journal of Medicine* 30:1344–49, 1981.

circulating antibody decreases, the animal is boosted with the antigen. Two to four days later, the spleen is removed and the nucleated cells are fused to a myeloma line with polyethylene glycol. The myeloma lines used for fusion lack the enzyme hypoxanthine phosphoribosyl transferase (HPRT), so that they die in medium containing hypoxanthine, aminopterin, and thymidine (HAT) (70). Most myeloma lines currently in use are variants that no longer synthesize the heavy or light immunoglobulin (Ig) chains of the original myeloma line (55, 121).

The fused cells are plated in HAT medium in 96-well microculture plates at concentrations that will result in one viable hybrid/well. The unfused myeloma cells die because they cannot survive in HAT medium, and the spleen cells eventually die because they cannot survive long-term culture under these conditions. By 2–3 weeks after the fusion, only hybrids between a normal spleen cell (which provides the HPRT, so that the hybrids will live in HAT medium) and a myeloma cell (which provides the ability to divide indefinitely in culture) are growing.

At this point the hybrids are screened for production of antibody to the desired antigen. Screening is conveniently done by radioimmunoassay (RIA) (39, 51) or enzyme-linked immunoassay (ELISA) (3, 145, 150). For example, the antigen is attached to a 96-well plastic plate and culture supernatant fluid from the hybrids added. After washing, antibody bound to the antigen is detected either with a radioactive (RIA) or enzyme-linked (ELISA) anti–mouse Ig antibody. The wells are then assayed for the presence of radioactivity (by counting) or the enzyme (by adding substrate and looking for the appearance of the appropriate color). ELISAs are usually more convenient and less expensive to perform than RIAs, especially since a wide variety of enzyme-linked antibodies and automated equipment that greatly facilitates the washing and reading of ELISA plates are now available.

Benefits of Monoclonal Antibodies

Monoclonal antibodies have many advantages over polyclonal antibodies. As mentioned above, once a useful hybridoma has been generated, the exact same antibody can be used by many different groups indefinitely, eliminating one source of differing results among laboratories. This is especially important for routine diagnostic reagents and for clinical and epidemiological studies. Large amounts of this antibody can be produced by growing the cells in mass culture, where they will secrete up to 100 $\mu g\ ml^{-1}$ of antibody, or by injecting them into a mouse to form an ascites tumor. Ascites fluids can contain up to 10 $mg\ ml^{-1}$ of antibody. The monoclonal antibodies can also be biosynthetically labeled by adding radioactive amino acids such as ^{35}S methionine or ^{75}Se selenomethionine (30, 46) to the culture medium, externally labeled with radioactive or fluorescent molecules, or attached to enzymes for use in ELISAs.

One crucial benefit of the hybridoma technology is that it can be used to generate "pure" antibodies from "impure" antigens. Because monoclonal antibodies are homogeneous (i.e. each antibody in the pool is identical to all the others) they recognize only one antigenic determinant. Thus, even if a multiantigenic preparation is used—such as cell membranes, intact cells, or virus infected cells—each monoclonal antibody will recognize only one of the many antigens presented to the animal. For example, immunization with whole cells has been used to generate hybridomas against: the murine and human major histocompatibility antigens (5, 20, 79, 90, 94), differentiation antigens on T lymphocytes (62, 75, 76, 102), transferrin receptors (43, 48, 93, 128), potential tumor-specific antigens (61, 69, 84, 103, 146), viral polypeptides that are presented on infected cell surfaces or are present within infected cells (71, 74), and parasite antigens (96, 98, 111, 119).

Of course, if the animal has been immunized with a mixture of antigens, it is essential to develop a screening assay that will identify only the desired antigenic determinant. For example, an initial screen for viral antigens could select for monoclonals that react with lysates of infected cells but not with uninfected cells. Monoclonals that are positive by this preliminary test could then be screened for reactivity with individual viral peptides by western blotting, virus neutralization, or some other more specific test.

Monoclonal antibodies also enable one to study small changes in a complex macromolecule or organism. Subtle changes in the sequence or conformation of an antigenic determinant are likely to be recognized and may even result in a complete loss of reactivity. As will be described later, such monoclonals will not only detect single amino acid substitutions but also make it possible to select or enrich for mutant antigens with primary sequence changes.

Yet another major advantage of monoclonal antibodies is the opportunity to obtain the best possible antibody for a particular use. If the antigen is very immunogenic, many monoclonal antibodies can be generated against it. The antibody with the most desirable characteristics can then be selected and used. Some properties that may affect the usefulness of an antibody are: its affinity and avidity for the antigen (how tightly it binds the antigen); whether it fixes complement; whether it binds to receptors for the Fc portion of Ig (Fc receptors) on phagocytic cells; and how stable it is in solution, especially after freezing and thawing or after purification. The affinity of the antigen binding is a function of the variable (V) region. The other properties are a function of the Ig constant (C) region (see Figure 2).

However, quite often only a few hybridomas producing the desired antibody are obtained from a fusion, particularly if the antigen is not very immunogenic or if only small amounts are available. Some antigens, for reasons still not completely understood, give rise to antibody responses dominated by one or a few classes of Ig heavy chains. It is therefore possible to identify a monoclonal

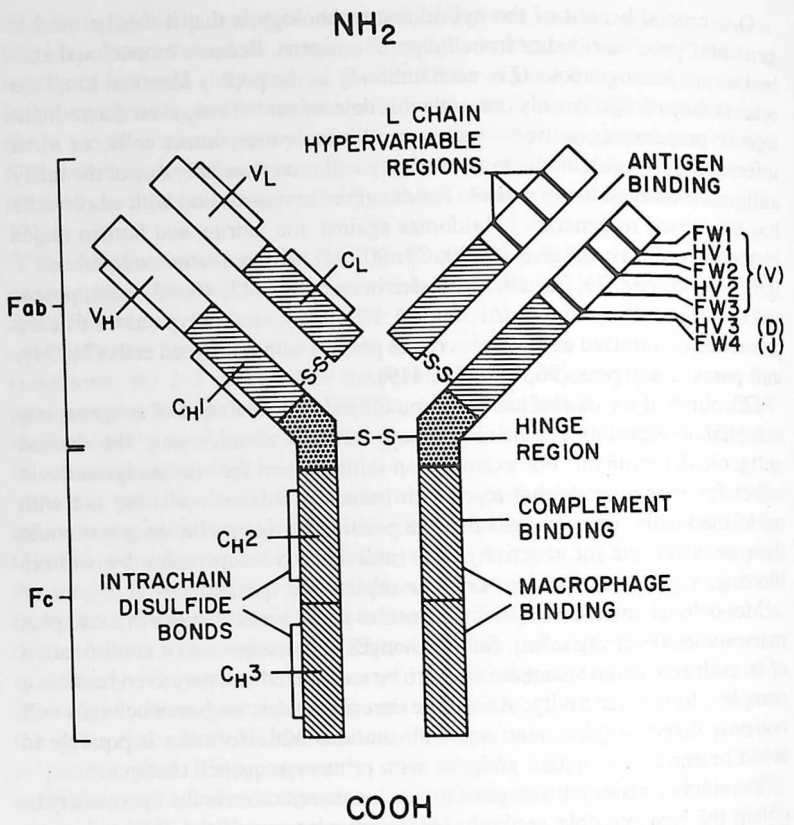


Figure 2 Structure of an antibody molecule. The different parts of the heavy-chain V region are indicated on the right, and the segments of the gene that encode them are shown in parentheses. Reproduced from *Science* 222:721–26, 1983, copyright 1983 by the AAAS.

antibody that has a useful antigen specificity and a high affinity, but that has an Ig heavy chain with undesirable properties.

There are two methods of obtaining a useful antibody from such a hybridoma. First, it is possible to identify rare subclones that still express their original V region and hence have the same antigen binding but have "switched" to express a different C region (27, 83, 99, 130). Such switching occurs during a normal immune response, with the heavy-chain class switches proceeding downstream (5' to 3') in the following order in the mouse: $\mu,\delta,\gamma^3,\gamma^1,\gamma^{2b},\gamma^{2a},\epsilon,\alpha$. Because each newly expressed C region has different biological functions, the switch variant may be more or less useful. Switch variants can be isolated sequentially at a frequency of 10^{-6} to 10^{-7} until the desired class is expressed (27, 83).

If the original hybridoma makes an IgG antibody but an IgM antibody is necessary, an IgG to IgM switch should be impossible because the process moves downstream (5' to 3'). Once a switch has occurred, the 5' C region genes are usually deleted (50, 110, 131), making an IgG to IgM switch even less likely. However, reverse switches have been reported in myelomas, although the precise mechanism is not known (83, 110).

Some properties that may complicate the use of antibodies, such as the formation of antigen-antibody complexes or the binding to Fc receptors, are inherent in the structure of all mouse IgG antibodies. These properties may be eliminated by using chemically or enzymatically produced fragments or by identifying deletion mutants that result in loss of a biological function. Yelton & Scharff (147) have generated such mutants from a hybridoma synthesizing an IgG2b antibody directed against the hapten azophenylarsonate. After mutagenesis, they found hybrids with large deletions in the heavy-chain constant region, in either the C_H2 or C_H3 domain (see Figure 2). These mutants lost the biological activities, such as Fc receptor binding and complement fixation, associated with the deleted part of the molecule, while retaining their antigen-binding ability.

In the near future it should be feasible to generate desired mutants by cloning the DNA encoding the heavy chain and altering the nucleotide sequence to give the desired biological properties, including antigen binding. The modified heavy-chain gene could then be transfected, along with the appropriate light-chain gene, into cells that would synthesize and secrete the Ig. Recent experiments by Oi et al (91) prove that Ig genes can be transfected into cells and expressed.

Technical Problems

A major problem encountered with the hybridoma technology is obtaining useful monoclonal antibodies against weakly immunogenic antigens. Such antigens may be highly conserved, have chemical structures that are not very immunogenic, or merely be available in very small amounts. It is possible to increase the immunogenicity of a molecule by chemically modifying it or by attaching it to a very immunogenic carrier. Both of these goals can be achieved by cross-linking the antigen to keyhole limpet hemocyanin (KLH) with gluteraldehyde (113). If an antigen is highly conserved, it may be possible to generate autoantibodies by using strains of mice that are subject to autoimmune diseases.

Another approach to this problem is to enrich for cells producing the desired antibody prior to fusion. This can be done in vivo by injecting spleen cells from an immunized donor into lethally irradiated recipients along with antigen. The spleen of the recipient is repopulated by the donor cells, and often contains a high percentage of antibody-forming cells that have been stimulated to replicate by antigen (38, 57, 123). Still another tactic is to incubate spleen cells in vitro

with antigen under conditions that induce the antigen-specific cells to proliferate while the other B cells die (38, 123). In both cases those stimulated cells are then fused.

Many other enrichment techniques exist in the literature. For example, antigen-binding cells can be adsorbed to antigen-coated dishes, columns, or sheep red blood cells, and then eluted. Up until now these techniques have not been particularly useful in making monoclonal antibodies, because even though they significantly enrich for cells producing a particular antibody, the absolute number of specific B cells recovered is small. Because fusion efficiencies are low (1 viable hybrid for every 2×10^5 B cells), it is difficult to obtain hybridomas. However, new techniques such as electrofusion (151) may overcome these problems and lead to dramatic changes in the technology.

The major other persisting problem in the hybridoma field is the difficulty of generating human hybridomas. For many clinical and diagnostic uses, human antibodies would be preferable to mouse antibodies. However, when human B lymphocytes are fused to mouse myeloma cells, the resulting hybrids lose human chromosomes so quickly that it takes a great deal of work to obtain a stable, antibody-producing hybridoma. Human myeloma cells are available for fusion and a number of lines have been tested for their suitability as fusion partners (1, 92). Although none have gained general acceptance, it seems likely that a useful line will eventually be found.

The generation of human hybridomas is further complicated by the fact that, for ethical reasons, humans cannot be hyperimmunized and boosted like mice. In vitro immunizations, mentioned earlier, may help overcome this particular limitation. Also, human spleen cells are not easily obtainable, and so peripheral B cells are usually used. These B cells are most likely in a different stage of differentiation or activation than the splenic B cells, and this may contribute to the difficulty of obtaining human hybridomas (64).

Monoclonal Antibodies Can Define Antigenic Structure

The availability of monoclonal antibodies and the potential of synthetic polypeptides as vaccines (2, 120) has lead to a reexamination of the nature of antigenic determinants. Many reviews have been written on this complex subject (2, 116, 117, 120, 134). A protein is viewed by the immune system as a large collection of antigenic determinants; an antigenic determinant, also called an epitope, is that very small part of the protein (or other molecule recognized by antibodies) that an antibody recognizes and binds via its antigen-combining site. Many epitopes can be localized to those parts of the protein that are on the outer surface of the folded molecule, particularly to regions that jut out, such as loops, carboxy termini, and turns between helices. Studying a protein's epitopes can provide much useful information on its three dimensional structure, on how it is organized into higher structures, and on the location of mutations.

An antigenic determinant that is created by a short linear sequence of amino acids and is relatively independent of their folding is termed a sequential determinant (116, 117). An antibody recognizing a sequential determinant should still bind to the protein even when it has been completely unfolded or denatured.

In contrast, antibodies against conformational determinants recognize a small, three-dimensional portion of the molecule (116, 117). Conformational determinants can be formed by a short sequence of amino acids contiguous in the linear sequence (a continuous conformational determinant) or by amino acids located in different parts of the linear sequence that have been brought together through the folding of the protein (discontinuous conformational determinants). Such discontinuous conformational determinants can also be constructed of amino acids from two different polypeptide chains that interact with each other. Because conformational determinants are a function of the protein's three-dimensional structure, they are usually lost when the protein is unfolded or denatured—but they may be recovered if the protein refolds. An antibody that binds the native protein but not the denatured protein probably recognizes a conformational determinant. Although sequential antigenic determinants are usually unaffected by changes elsewhere in the protein, conformational antigenic determinants can be affected by any change that alters the three-dimensional structure of the molecule.

Typically, only a limited number of antigenic determinants are detected when the antigenic structure of a protein is analyzed with polyclonal antibodies. These determinants are strongly immunogenic, perhaps because they are more exposed on the surface of the protein, so that most of the antibodies are directed against them. The few antibodies that recognize less immunogenic determinants are usually obscured by the more numerous antibodies to stronger, "immunodominant" determinants.

By contrast, once a monoclonal antibody against a nonimmunodominant determinant is identified, its reactivity can be analyzed as effectively as that of a monoclonal against an immunodominant determinant. Therefore, a better estimate can be obtained of the number and types of antigenic determinants on a protein. Because monoclonal antibodies make it possible to study many more determinants than polyclonal antisera, small differences between proteins can be more easily detected. Such antibodies have proved useful in making distinctions that were previously impossible and identifying single amino acid substitutions in protein antigens (25, 107, 108, 115, 140, 149).

Antigenic determinants can be localized in the protein's structure if its amino acid sequence and three-dimensional structure are known. Fragments of the protein and synthetic peptides containing short sequences of the protein are useful in determining what part of the protein sequence constitutes a particular determinant. Localization of antigenic determinants can also be done with

proteins that have only a few sequence changes from the protein under study. Such proteins may be mutants or proteins related by evolution. Once the amino acids involved in a particular determinant are known, the determinant can be located on the three-dimensional structure.

ANALYSIS OF VIRAL ANTIGENS

In the previous sections, we have noted that monoclonal antibodies provide a unique tool for identifying small amino acid substitutions in proteins and for determining the locations of these changes. Such applications of monoclonal antibodies have been employed in many systems. However, the use of monoclonals in the classification and diagnosis of viral diseases and, in particular, in the dissection of the antigenic structure of influenza virus best illustrates the enormous potential of this approach.

Analysis of Influenza Virus Epitopes

Influenza virus is very immunogenic in mice. A large number of monoclonal antibodies were generated against the viral hemagglutinin by Koprowski and his colleagues (60), and subsequently by others, soon after the hybridoma technology became available. The influenza hemagglutinin is a glycoprotein that is exposed on the surface of the virus and mediates the adsorption and penetration of the virus. Antibodies directed against it can neutralize the virus.

In nature frequent changes occur in the structure of the hemagglutinin, contributing to the notorious antigenic instability of influenza virus. When these changes are subtle and occur between pandemics they are called antigenic drift. When the changes are more dramatic, as occurs during pandemics, they are termed antigenic shifts (31). Both types of changes are usually associated with the multiple amino acid substitutions (136).

Many antigenic variants of influenza have been identified and preserved because of their medical importance. These strains have been serologically characterized, originally with conventional antisera and more recently with monoclonal antibodies. The hemagglutinins of some of the more important strains have been sequenced (17, 86, 135, 144). Furthermore, the three-dimensional structure of one influenza hemagglutinin has been determined (143).

Monoclonal antibodies against certain parts of the hemagglutinin prevent the virus from infecting cells. Therefore, when cloned virus stocks are incubated with such antibodies, rare mutant viruses are recovered that can propagate in the presence of the monoclonal antibody (42, 65, 66, 72, 148). The selection of such mutants is possible with monoclonal antibodies, but not with most antisera. Because conventional antisera contain antibodies against more than one antigenic determinant, multiple mutations in the hemagglutinin would have to occur for the virus to escape neutralization. In contrast, a single mutation could render the virus nonreactive with an individual monoclonal antibody.

The frequency of mutant particles that no longer react with one monoclonal antibody was estimated to be 10^{-5} (42, 65, 66, 72, 148). When virus stocks containing 10^8 infectious units were exposed to a pseudopolyclonal antiserum composed of two unrelated monoclonals, mutants could not be isolated, as would be predicted from the frequency of point mutations. Most of these in vitro mutants have single or a very few amino acid substitutions while the naturally occuring mutants usually have many amino acid changes (17, 42, 65, 66, 72, 86, 148). As would be expected, when the same monoclonal antibody is used to select variants from independently cloned virus stocks, the variants often have identical amino acid substitutions or substitutions in amino acids that lie close together in the three-dimensional structure (17, 65, 86).

The details of the studies on influenza virus have been reviewed by the workers in the field (17, 149), so we will only summarize some of the relevant conclusions. First, as anticipated, monoclonal antibodies can detect differences that are not detected when conventional antisera are used (42, 137, 138). Mutants isolated in vitro from standard strains are often not distinguished with the available panels of polyclonal antisera generated by immunization or obtained following infection. Some of the most discriminating antisera were obtained by immunizing ferrets, and even these seldom distinguished the in vitro mutants from the parent hemagglutinin (138). Not surprisingly, an in vitro mutant does not react with the monoclonal used to select it but reacts well with other unrelated antihemagglutinin monoclonals.

Of more importance, when panels of monoclonals were used to examine strains that arose between pandemic years, antigenic drift was detected that had not been detected with polyclonal antisera (42, 137, 138). Monoclonals also revealed differences between viruses isolated in different parts of the world during a single epidemic (138). These studies indicate that monoclonals will be invaluable in epidemiological studies.

The restricted reactivity of monoclonals also makes them very useful for detecting shared specificities. Some influenza strains that show minimal cross-reactivity with antisera react almost identically with a few monoclonal antibodies, suggesting that these virus isolates had a common origin (149).

Mapping of Influenza Virus Antigenic Determinants

The primary sequence differences between epidemic strains have been used to map the antigenic domains of the influenza hemagglutinin (142). These studies have been greatly refined by using monoclonal antibodies. Cross-competition experiments were done by determining whether each member of a panel of monoclonals could interfere with the binding to influenza hemagglutinin of each other panel member (14, 17, 132, 138, 149). Competing monoclonals are

considered, at least initially, to react with the same or structurally closely-linked antigenic determinants. These studies confirmed the existence of at least four antigenic domains on the hemagglutinin molecule (14, 17, 132, 138, 149).

Such cross-competition experiments can be misleading if two monoclonals bind with very different affinities (or avidities), so that one will not inhibit the other even if it is present in considerable excess. Another source of error occurs if the binding of one antibody causes a conformational change in the antigen that creates or destroys an antigenic determinant that is recognized by a second monoclonal (73). The occurrence of either event can be recognized by using each member of the panel to inhibit all other members.

The presence of at least four antigenic domains was futher confirmed by isolating in vitro mutants and examining how their amino acid substitutions affected their binding by monoclonals that were not used to select them (17, 42, 65, 66, 72, 86, 148). Mutations in one domain did not affect the reactivity of monoclonals mapping to the other domains, suggesting that the domains were distinct and did not interact.

Even though 34 mutants and 58 monoclonal antibodies were used to generate this operational antigenic map of 4 distinct antigenic domains, the map appeared to be incomplete, because 8 additional monoclonals reacted equally well with all of the mutants and the parental hemagglutinin (17). When these eight monoclonals were used to select additional mutants, more antigenically unique mutants were identified. Analysis of these mutants revealed that one of four existing domains could be subdivided into two closely linked but distinguishable domains. Some of the other mutants revealed additional interrelationships between existing domains (17). While most of the monoclonal antibodies still defined distinct antigenic determinants, these findings and similar studies with larger numbers of monoclonals and hemagglutinins from other strains of influenza raise the possibility that a continuous antigenic map could be established if enough monoclonals were tested (132).

Most of the antigenic determinants were discontinuous conformational determinants in that amino acids from distant parts of the linear sequence contributed to each antigenic domain. Similarly, amino acids that were closely linked in the linear sequence often participated in different determinants. Further, all but one of the amino acids found to contribute to antigenic determinants were exposed on the surface of the hemagglutinin (17).

There are a number of other interesting points arising from the studies on the influenza hemagglutinin. Two hemagglutinins from distinctly different serotypes of the virus have been studied (17, 142). Each of the hemagglutinins has an antigenic domain that is not serologically detected on the other. Each nonantigenic domain is glycosylated, while the comparable domain on the other hemagglutinin (which is antigenic) is not glycosylated. This suggests that the oligosaccharide chain may protect the domain from recognition by the

immune system of the host (17). A second point is that many of the epitopes recognized by monoclonal antibodies show structural and antigenic variation in nature (17, 86, 142, 149). These epitopes may be more immunogenic in both mouse and man than other exposed sites on the hemagglutinin and thus subject to in vivo selection of mutants.

Identification of Influenza Virus Epitopes with Synthetic Peptides

Another approach for identifying epitopes is to examine synthetic polypeptides that are expected to contain linear antigenic determinants for their ability to inhibit binding of both polyclonal and monoclonal antibodies to the intact molecule. Biophysical studies have suggested that short peptides fold into conformations that are different than those which the same linear sequence acquires in the intact molecule. This assumption apparently was confirmed when synthetic peptides were used to inhibit the binding of conventional antibodies to intact proteins. In one study, competition experiments suggested that no more than 1 in 5000 peptide molecules were in a conformation corresponding to that of the intact molecule (134). Such peptides were thus assumed to be poor immunogens for generating antibodies that would react with native structures. It was therefore surprising that immunization with synthetic peptides attached to immunogenic carriers stimulates the production of antibodies that reacted with intact molecules (2, 68, 115).

Studies using synthetic peptides to identify antigenic determinants have been carried out with the influenza hemagglutinin. In one illustrative study, 20 peptides, each containing 9–36 amino acids and representing 75% of the amino acid sequence of the hemagglutinin peptide, were synthesized, attached to an immunogenic carrier, and used to immunize rabbits (45). None of these peptides reacted significantly with conventional antihemagglutinin antisera, suggesting that the peptides were not in a native configuration. Nevertheless, 18 of the 20 antipeptide polyclonal antisera generated recognized the intact hemagglutinin. Interestingly, most reacted with sites that were not in the antigenic domains defined by monoclonals or antisera generated by immunization with intact hemagglutinin (45). This is not only perplexing but could have practical implications, because synthetic peptides are being examined for their usefulness as vaccines.

It is generally thought that sites that are not immunogenic or only weakly so are either not well exposed on the surface or have a chemical structure that does not stimulate the immune response. However, it is also possible that some of these sites cross-react with some autoantigen and that the individual's response to them may be specifically suppressed so as to prevent autoimmune disease. If this were true, and the synthetic peptide was able to induce antibodies against them, then vaccination with such a peptide might be dangerous.

Because it was possible that only a small percentage of the antibodies in these antipeptide antisera reacted with the native protein, one of the synthetic peptides (containing 36 amino acids) was used to generate monoclonal antibodies. Twenty-one monoclonals that reacted with the immunizing synthetic polypeptide were identified; sixteen of these reacted with the intact hemagglutinin (87). This result suggests that the majority of antibodies resulting from immunizations with synthetic peptide will react with the native molecule. Similar experiments with synthetic peptides from other virus and transplantation antigens confirmed this finding (87).

Even more astonishing is the observation that synthetic peptides containing only 5–6 amino acids, when injected free (unattached to carriers), are immunogenic and can be used as immunogens for generating monoclonal antibodies that react well with native proteins (115). The success of immunization with synthetic peptides is of considerable conceptual and practical importance and suggests the possibility of inducing immunity by vaccinating with synthetic peptides (68). The effectiveness of this approach remains unexplained. The immune response may recognize and preferentially respond to native configurations, or the antigen-presenting cells may fix peptides into native configurations (87). However, there is no direct evidence to support either of these theories. These studies clearly demonstrate that a great deal has yet to be learned about immune recognition and responsiveness.

Analysis of Other Viruses

Similar, although less extensive, studies to those described for influenza virus have been carried out with a number of other viruses. For example, Nowinski and his colleagues generated monoclonal antibodies against a murine leukemia virus gp70 envelope protein. Some of these monoclonals neutralized the virus while others did not, implying multiple antigenic domains (88, 127). This was confirmed when it was found that nonneutralizing antibodies were effective in lysing infected cells with gp70 on their surface, while the neutralizing antibodies were not (89).

Fields & Joklik and their colleagues generated monoclonal antibodies against the reovirus Type 3 hemagglutinin and also found multiple functional domains (16, 47, 67). Some monoclonal antibodies neutralized the virus, others inhibited the hemagglutination mediated by the viral protein, while others could, to varying degrees, both neutralize and inhibit hemagglutination. Mutant viruses were selected that were no longer neutralized by a monoclonal antibody. These replicated well on L cells but when they were injected in the brains of mice, their neurovirulence was markedly reduced (126). These studies not only indicate that an antigenic domain that is recognized by neutralizing antibody plays an important role in tissue tropism, but also suggests a strategy for generating attenuated virus (126).

Neutralizing antibodies have also been used to estimate the number of epitopes through which poliovirus can be neutralized (33, 78) and to show that in poliovirus Type 1 such determinants can be found on each of the three exposed polio polypeptide chains (33). As with influenza virus, variants that had lost the ability to react with neutralizing monoclonals arose frequently in culture, even though in nature polio is not considered a particularly unstable virus. Synthetic peptides that can elicit neutralizing antibody have also been generated (34).

The use of monoclonal antibodies and synthetic peptides will certainly lead to a better understanding of the antigenic properties of viruses and other microorganisms, and to new approaches to inducing immunity. The benefits of monoclonals in diagnosis are already obvious. Viruses such as rabies and measles, which were thought to be antigenically very stable, have been shown to be antigenically heterogeneous (12, 140). Using monoclonals it has been possible to carry out epidemiological studies (141) that were not possible with polyclonal antisera. If one adds the benefit of more restricted specificity to the availability of large amounts of the exact same antibody indefinitely, it becomes obvious why monoclonal antibodies are and will continue to be such powerful tools.

ANALYSIS AND MANIPULATION OF IMMUNOGLOBULIN IDIOTYPES

Antibody Diversity

It is implicit in all of the previous sections that antibody-combining sites can be generated that recognize each of the small changes that occur in the antigenic domains of viral or other antigens. In fact, examining the specificity and structure of monoclonal antibodies has led to a new appreciation of the extent of antibody sequence diversity, and of the structural and genetic basis of this diversity. Just as with viral antigens, monoclonal antibodies can be obtained that react with restricted parts of the immunoglobulin molecule; these antibodies are powerful tools for identifying and manipulating the microheterogeneity of antibodies.

A typical antibody molecule is shown in Figure 2. The basic structure consists of two heavy (H) chains and two light (L) chains. Each H and L chain contains a variable (V) region and a constant (C) region. The V region consists of about 110 NH_2 terminal amino acids, with the rest of each chain constituting the C region. The V regions fold to create the antigen-binding site and contain most of the sequence diversity found in antibodies. The actual contact residues with antigen are located in three small segments of the V_H and light-chain V region; because they contain most of the sequence diversity, they are called the

hypervariable (HV) sequences. The remaining, less variable sequences are termed the framework (FW) sequences.

The V and C regions are encoded separately in the genome, and the V-region gene is rearranged to lie close to the C-region genes during B-cell differentiation. Another segment, called the joining (J) segment, lies between V and C in the rearranged DNA and actually forms the join between the two regions in the protein. Heavy chains have an additional segment, the diversity (D) segment, that is rearranged to lie between the V and J genes. The D and J segments form part of the third HV region (Figure 2). Furthermore, in the course of the VDJ or VJ rearrangements, bases can be inserted or deleted, producing additional "junctional" diversity in the third HV region. This rearrangement process creates many different antibody sequences from a limited amount of genetic material consisting of a few hundred V genes, twenty or so D genes, and four J genes (reviewed in 50, 131).

Both polyclonal antibodies and monoclonal antibodies have been used extensively to study the V regions of antibody molecules. The antigenic determinants on a particular antibody's V region define that antibody's idiotype. Individual antigenic determinants, called idiotopes, can be located in the hypervariable or framework regions of the V region, and can be either sequential or conformational determinants. The V regions, D, and J segments all appear to play a role in forming various idiotopes.

Idiotopes encoded by germ-line V-region genes are usually detected in all individuals of a particular strain, and are termed public or cross-reactive idiotopes. Idiotopes reflecting somatic mutation or infrequent junctional changes are expressed only in occasional individual animals or in one or a few monoclonal antibodies, and are called private idiotopes. Some idiotopes are in or near the antigen binding site, and antibodies against them inhibit antigen binding, while other idiotopes are outside the site, and antibodies recognizing them have no effect on antigen binding. Antibinding-site antibodies usually recognize hypervariable region residues. The expression and function of idiotypes has been reviewed recently in great detail (100).

Polyclonal anti-idiotypic antisera are generated by immunizing animals, usually of another strain or species, with the antibody under study. The resulting antisera often contain antibodies against both the constant and variable regions. Therefore, the antisera must be absorbed with other immunoglobulins that have a different V region to remove any serum antibodies that recognize non–V-region antigenic determinants. The absorbed antiserum is then called an anti-idiotypic antiserum. Because such antisera are polyclonal, they will contain antibodies against both binding site and nonbinding-site idiotopes, and the anti-idiotopic antibodies may differ from antiserum to antiserum.

Monoclonal anti-idiotypic antibodies are raised by immunizing mice or rats with the antibody expressing the idiotype and then generating hybridomas.

Hybrids producing antibodies that react with the immunizing antibody, but not with other Igs that have the same class of heavy- and light-chain class but a different V region, are presumed anti-idiotypic antibodies. Because monoclonal antibodies recognize only one epitope, they are actually anti-idiotope antibodies.

Analysis With Anti-Idiotypic Antibodies

Idiotopes recognized by polyclonal anti-idiotypic antisera have been localized by using Ig with one or a few amino acid changes in their V region (9, 22, 114). The absorption procedure used to make specific anti-idiotype antisera may selectively remove antibodies recognizing conformational determinants, leaving antibodies that bind sequential determinants, so that localization of idiotopes is readily accomplished. Similar experiments using monoclonal anti-idiotope antibodies have been unable, in several cases, to localize the idiotopes (9, 22, 95, 114). For example, a monoclonal may distinguish antibodies with different D segments without defining a particular amino acid sequence in the D segments it recognizes (22, 85, 114).

This difficulty in mapping antigenic determinants with monoclonals may reflect the fact that many idiotopes appear to be conformational determinants, requiring the presence of the correct heavy and light chain. An unusual conformational determinant has been described by Morahan, Berek & Miller (81) who reported a monoclonal antibody that reacts with the T15 V_H region [which binds phosphorylcholine (PC)] only when it is associated with the IgA constant region and not when it is associated with IgM or IgG constant regions. Several cases have been found where a single amino acid substitution could cause an Ig to lose or gain an idiotope (100, 129). Such idiotopes may be sequential determinants or may be conformational determinants that have been altered structurally by the single amino acid change.

Studies of the amino acid or nucleotide sequences of monoclonal antibodies have revealed the importance of somatic mutation in generating antibody diversity. This was first appreciated in studies by Weigert, Cesari & Cohn, that suggested that somatic mutation contributes to the sequence diversity of lambda (λ) light chains produced by mouse myeloma cells (139). Tonegawa and his colleagues confirmed the above observations by demonstrating that there is only one V_L germ-line gene for λ_1 light chains, and that this V_L sequence changed in myelomas producing the variant light chains (11). Subsequently, much information about idiotype expression and somatic mutation has been obtained in systems using idiotypes expressed by antibodies that form a major portion of the immune response to a hapten, and which are encoded by one or a few germ-line genes. Such idiotypes are found in the response of mice to PC, azophenylarsonate (ARS), ($\alpha 1 \rightarrow 3$) dextran, (4-hydroxy-3-nitro phenyl) acetyl (NP), and 2-phenyloxazolone (reviewed in 100; see also 52, 53).

Gearhart and her colleagues have studied 11 IgM and 9 IgG anti-PC mono-

clonal antibodies (40). The V_H regions of the IgM antibodies all had the same NH_2 terminal sequence, that of the germ-line gene encoding the T15 V_H region (107). The IgG antibodies were all products of the same germ-line gene but contained one or a few amino acid substitutions. Changes could be found in both the HV and the FW regions. These changes can be best explained by somatic mutations (26, 40, 58), although alternative mechanisms such as gene conversion (see below) cannot be ruled out. Because IgM is made earlier in B-cell differentiation than IgG, these results suggested that the mechanism that produced base changes was either turned on during class switching (from IgM to IgG production) or that mutations accumulated during the expansion of B-cell clones. Subsequent studies suggest that the latter is true (52, 53, 104).

Studies on other idiotypic families encoded by a single germ-line gene have confirmed that many antibodies are derived by a few base changes from the germ-line sequence. Many of the ARS-binding antibodies expressing the cross-reactive idiotype (CRI) (77, 122) are derived from a single germ-line V_H gene but differ from it by a few amino acid substitutions in the HV or FW parts of the V region (77, 122, 124). Because no two of the IgG anti-PC antibodies analyzed by Gearhart et al (26, 40, 58) were identical, and there are few repeats in the anti-ARS antibodies (77, 122, 124) and in other systems that have been studied (13, 15, 29, 52, 53, 100, 109), there must be even more sequence diversity than was previously suspected. It is now estimated that an individual produces as many as 10^9 different antibodies in a lifetime.

The ability to sample the idiotypic repertoire through the analysis of monoclonal antibodies has been very useful, but this approach has some limitations. Such monoclonal antibodies are the product not only of B-cell differentiation, but also of selection by antigen and T cells. These immunoregulatory pressures probably select for variants with higher affinity for the antigen then encoded in the germ-line sequence. In fact, all of the monoclonal antibodies shown to differ from the germ-line sequence by Gearhart and her colleagues (105) and by Rothstein and Gefter (105a) have higher affinities for the antigen than antibodies with the germ-line sequence. Because such selective pressures are operating, it is difficult to determine the exact frequency of V region mutations and to recover all the mutants (because the lower affinity mutants will not be expanded).

The instability of Ig genes can also be examined in cultured myeloma and hybridoma cells, using techniques that have proven very useful in bacterial genetics. This approach is feasible because we and others have developed techniques to identify somatic cell mutants of Ig producing cell lines (82). Mutants in the V-region genes occur in vitro at very high frequencies of 0.01% to 1% (82). Because cultured cell lines are clearly different from normal cells—they carry viruses, grow continuously in culture, and can form tumors in animals—information obtained from such cells must be carefully interpreted.

However, the high rate of spontaneous mutation appears to be localized to the Ig genes because genes encoding other proteins, such as thymidine kinase and hypoxanthine phosphoribosyl transferase, undergo mutations at the expected frequencies of 10^{-6} to 10^{-7} (6).

Our studies have concentrated on the V_H region gene of anti-PC antibodies of the T15 idiotype as it is expressed in the S107 myeloma and in hybridomas. When S107 cells are cloned in soft agarose and overlaid with antigen [PC coupled to a large protein carrier such as keyhole limpet hemacyanin (KLH)], the secreted antibody reacts with the antigen to form antigen-antibody precipitates around the cells (23, 24). Variant clones that no longer bind antigen, or no longer secrete antibody, are not surrounded by such immunoprecipitates (24, 25, 108). These variants can be recovered from the agarose and characterized to determine if they are secreting a nonantigen-binding antibody.

In the seven mutants analyzed so far, the defect in antigen binding resides in the heavy chain (24, 25, 108). Two of these mutants have been sequenced and shown to have a single amino acid change from the parental sequence. In the U_4 mutant an alanine has replaced the glutamic acid at residue 35, and in the U_1 mutant an alanine has replaced the aspartic acid in the fifth residue of the J segment (25, 108). It is interesting that a survey of myelomas expressing this same J segment revealed a number of somatic mutations at this residue, suggesting that it may be unusually stable (44).

The U_4 mutation is particularly interesting with respect to both the structural basis of antigen binding and the genetic expression of the T15 family of V_H region genes. Because the heavy chain has four residues that make contact with hapten and the light chain has one contact residue (28), it was surprising that the change of one amino acid resulted in a greatly decreased affinity for antigen. However, the three dimensional structure suggests that the hydrogen bond between the glutamic acid at residue 35 of the heavy chain and the tyrosine at residue 94 of the light chain stabilizes the conformation of the antigen-binding site (28). This also explains why only this particular member of the T15 family of cross-hybridizing V_H-region genes is usually expressed in PC-binding antibodies from BALB/c mice; none of the others code for a glutamic acid at residue 35 (26). Finally, this is a product of the germ line that is "degenerate" in that it no longer binds PC. Although it is not clear whether it will react with another antigen or represents a waste product, it is a gene product that would be missed in screening for PC binding hybridomas.

The mechanism responsible for the high frequency of somatic mutation in Ig V region genes is not known, although the types of mutations suggest that simple recombination is not the answer. Analysis of the types of base changes that occur in monoclonals does not show any significant dominance of transitions, transversions, or particular base changes (40, 58, 77, 97, 118, 122). Gearhart & Bogenhagen have reported that the base changes occur in clusters,

and they suggested that an error-prone polymerase is involved (41). On the other hand, Rajewsky and his colleagues have isolated somatic mutants in vitro from an anti-NP hybridoma, and they have found one mutant that differs from the presumed germ-line sequence by ten closely linked amino acids (15, 29). A likely explanation for this mutant is that it arose through the interaction of two closely related V-region genes and that a gene conversion-like mechanism could play a role in generating antibody diversity (4, 15, 29).

Manipulation of the Immune Response with Anti-Idiotype Antibodies

Anti-idiotype antibodies have been used to enhance or suppress expression of particular idiotypes in mice (reviewed in 100). Polyclonal anti-idiotypic antibodies were originally used, but conflicting results were sometimes obtained. With the development of monoclonal antibodies, such experiments could be done with antibodies directed against individual public or private idiotopes.

Newborn mice are exquisitely sensitive to suppression by polyclonal or monoclonal anti-idiotype antibodies. As little as 1 μg of antibody can suppress the expresion of that idiotype (or idiotope) for several months or longer. Such mice will make an antibody response devoid of the idiotope when immunized with the antigen recognized by the idiotype bearing Ig (8, 10, 19, 56, 100). Neonatal suppression with individual monoclonal antibodies recognizing different idiotopes of the T15 idiotype usually results in the complete suppression of the T15 idiotype (8, 10, 19, 56, 100, R. R. Pollock, J.-L. Teillaud & M. D. Scharff, unpublished results). However, a few antibodies that express some, but not all, of the T15 idiotopes have been detected, even in mice suppressed with polyclonal anti-idiotypic antibodies (10, 19, 56). Monoclonal antibodies generated from these suppressed mice can be analyzed to determine whether they are the product of a different germ-line gene or are derived by somatic mutation from a single germ-line gene.

Anti-idiotype antibodies will also suppress idiotype expression in adult mice, providing larger amounts of anti-idiotype (or anti-idiotope) antibodies are used (10–100 μg). This suppression tends to be shorter lived than the suppression obtained in neonatal mice, and dissociation of idiotopes is more readily observed (56, 106). If adult mice are injected with small amounts of anti-idiotypic antibodies (10–100 ng), the expression of the idiotype can be enhanced (100).

Studies of this kind were originally complicated by results suggesting that the class of the anti-idiotypic antibody determined whether suppression or enhancement of the idiotype occurred (100). However, Muller & Rajewsky (83) took a monoclonal anti-idiotope antibody of the IgG_1 class and derived from it in culture a series of switch mutants that contained the exact same V region, but now associated with IgG_{2b} or IgG_{2a} constant regions. These sets of anti-idiotope antibodies were then used to study enhancement and suppression of the idiotype. This elegant set of experiments showed that the subclass of the antibody was not important, and that the earlier results were probably due to different affinities of the anti-idiotypic antibodies (83, 100). It also illustrates how monoclonal antibodies can be tailored to provide more useful reagents.

The mechanism by which such enhancement or suppression occurs is not well understood. In both cases, the B cells and helper or suppressor T cells have been shown to be directly involved (19, 100).

This ability to manipulate the idiotypes expressed during an immune response may prove useful in the future. For example, in many autoimmune diseases such as myaesthenia gravis and lupus erythematosis, the autoantibodies often show a predominant idiotype (32, 125). It may be possible to suppress these autoantibodies by giving the patient an anti-idiotype antibody directed against his or her predominant idiotype. Such a procedure may be complicated by the fact that, as discussed in the final section of this paper, anti-idiotype antibodies can elicit anti-(anti-idiotype) antibodies, and some of the anti-(anti-idiotype) antibodies can resemble the idiotype.

MONOCLONAL ANTIBODIES AS PROTECTIVE AGENTS

Immunization with Anti-Idiotype Antibodies

In the preceding sections we discussed how monoclonal antibodies can be used to detect point mutations in viral proteins. In addition, we have described how the structural analysis of monoclonal antibodies has revealed the enormous sequence diversity in the antigen-binding sites of the immunoglobulin molecule and how anti-idiotypic antibodies can be generated that recognize the sequences that provide some of this diversity. Such anti-idiotypic antibodies have been used to induce protective antibodies in animals that have never seen the pathogen.

The procedure for doing this is practically rather complex but conceptually simple (Figure 3) (18, 133). A monoclonal antibody (ab-1) that can neutralize an infectious agent is identified. Its binding site must be complementary in structure to an antigenic determinant on the infectous agent that is essential for infectivity. A second monoclonal (ab-2) can then be generated that is anti-idiotypic and reacts with the binding site of ab-1. Because the binding site of ab-2 is complementary to the binding site of ab-1, it should be identical, or at least very similar to, the antigenic determinant on the infectious agent. In fact, ab-2 can bind to viral receptors and in some cases block the adsorption of virus (54).

If ab-2 is used to immunize a recipient, the recipient will make anti-(ab-2 anti-idiotypic antibody) antibodies (ab-3). Ab-3 antibodies that recognize the

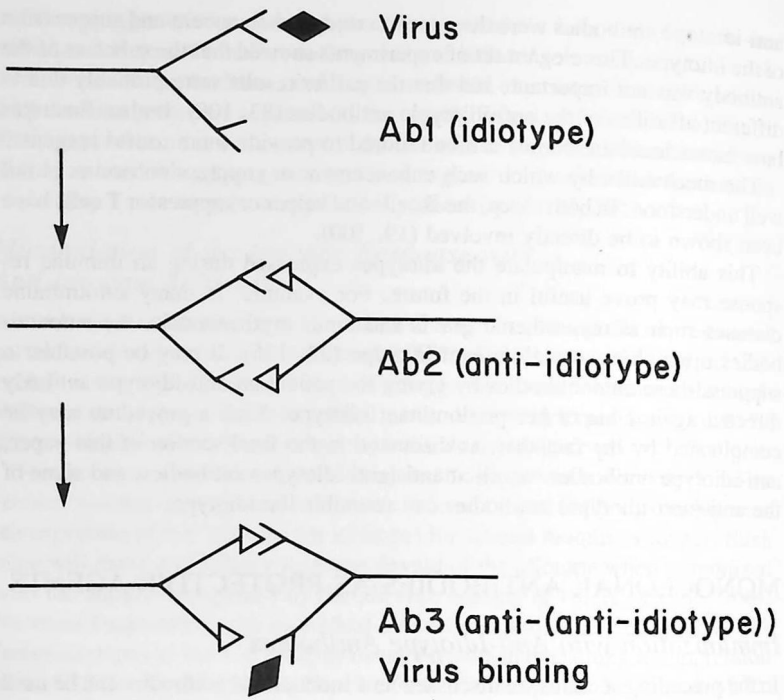


Figure 3 Induction of antigen-specific antibodies by anti-idiotypic antibodies. Ab-1 is an antibody specific for a given antigen, such as a viral antigenic determinant. Anti-idiotypic antibodies directed against ab-1 are termed ab-2 antibodies. Ab-3 antibodies are directed against ab-2 antibodies, and therefore are anti-(anti-idiotypic) antibodies. If an ab-2 antibody recognizes the antigen binding site of ab-1, ab-3 antibodies specific for the antigen binding site of ab-2 should bind the antigenic determinant recognized by ab-1.

antigen-binding site of ab-2 should neutralize the infectious microorganism. Thus an antibody can be used to induce neutralizing antibodies against a microorganism that either is itself not readily available or is dangerous to use. In a sense, ab-2 is an attenuated microorganism. This tactic has been used in animals to produce antibodies against the acetylcholine receptor (21), some tumor antigens (37), some viruses (35, 101), and trypanosomes (111, 112).

Sacks and his colleagues (111, 112) have shown that anti-idiotypic antibodies can induce protective immunity to African trypanosomiasis in mice. They generated three monoclonal antibodies, all of which reacted with the major variant surface glycoprotein of a cloned mutant of *Trypanosoma rhodesiense* and protected mice against infection by parasites bearing this variant glycoprotein (although parasites expressing new variant glycoproteins could appear). Polyclonal anti-idiotypic antibodies were prepared against each

monoclonal antibody and injected into mice. Antibodies directed against one of the monoclonal antibodies induced protective immunity in mice, while antibodies directed against the other two monoclonal antibodies did not. All three groups of anti-idiotypic antibodies increased the expression of the corresponding idiotype in the sera of recipient mice. However, when these anti-idiotypic antibodies were tested for their ability to block binding by the monoclonal antibodies to the variant glycoprotein, only the group of anti-idiotypic antibodies that conferred protective immunity significantly blocked antigen binding.

Neutralizing antibodies directed against rabies virus could be induced in mice by anti-idiotypic antibodies (101). Five monoclonal antibodies specific for the rabies virus glycoprotein, all but one of which strongly neutralized rabies virus infectivity, were used to generate polyclonal anti-idiotypic antibodies. Three of the anti-idiotypic antibodies inhibited antigen binding by the respective idiotype-expressing monoclonal. Two of these three anti-idiotypic antibodies, when injected into mice, induced antibodies that neutralized rabies virus. Interestingly, one of the two effective antibodies was directed against the one monoclonal antibody that did not strongly neutralize rabies virus. These studies suggest that immunizing with anti-idiotypic antibodies may prove to be clinically useful and confirm that the effective anti-idiotypic antibodies need to be specific for the binding site of the idiotype-expressing antibody.

Ertl, Finberg, and their colleagues have shown that a monoclonal anti-idiotypic antibody can induce a specific response at both the B-cell and the T-cell level (35, 35a). Their anti-idiotypic antibody is unusual because it was raised against a cloned, Sendai virus–specific, helper T-cell line. They term it an anti-idiotypic antibody because it recognizes the cloned Sendai virus–specific T cells, and a fairly high percentage of Sendai virus–specific T cells, but not T cells from unimmunized mice or T cells directed against unrelated antigens.

When this monoclonal anti-idiotypic antibody was injected into mice, they made measurable amounts of Sendai-specific antibodies, although the amount of antibody was usually less than that stimulated by the virus itself. Mice receiving the anti-idiotype also had Sendai virus–specific T cells; both Sendai virus–specific delayed-type hypersensitivity responses and cytotoxic T cells that would lyse Sendai virus–infected cells were observed. Although the relationship of anti-idiotypic antibodies generated against T cells and anti-idiotypic antibodies raised against Igs is not resolved, these studies suggest that anti-idiotypic antibodies can induce T- and B-cell immunity.

There are serious problems with applying this approach to human immunization. If ab-2 from a mouse or other species is used, the recipient will make antibodies against many parts of the mouse immunoglobulin in addition to its binding site. It would therefore be necessary to generate each of the successive

monoclonals in man. Also, many of the anti-(anti-idiotypic) antibodies (ab-3s) might be against idiotypes outside of the binding site. Even if this were not a problem, the ab-3s are antoantibodies and may not be made in very large amounts or persist for reasonable periods of time.

There are many less esoteric applications of monoclonal antibodies for protecting individuals against infectious agents or toxic substances. For example, monoclonal antibodies could be used to passively immunize recipients.

Passive Immunization

Monoclonal antibodies may prove useful for passive immunization against viruses, bacteria, or parasites in patients with ongoing infections. Potential patients would be people who do not have time to develop an adequate immune response, or individuals with a defective immune system. Such antibodies would act very specifically, and should have fewer side effects than many drugs. In fact, specific antibodies can be conjugated to different drugs (80), so that the drug is specifically delivered to the invading organism. In effect, the local concentration of the drug is increased while the concentration of the drug elsewhere remains minimal. Monoclonal antibodies, appropriately labeled, may also prove useful in localizing infections by radioimaging techniques.

There are several problems with passive immunization. A major one is that human monoclonals are not readily available. If a nonhuman Ig is injected into a human, the patient will probably make an immune response to the Ig. If large amounts of the monoclonal antibody are administered initially, or if the monoclonal antibody is given again, the patient's circulating antibodies will neutralize the injected monoclonals, and the resulting immune complexes could cause acute or chronic allergic reactions. The major limitation of this approach, however, is that the protection it conveys will be short lived, and no priming for a secondary immune response will occur.

Vaccine Preparation

Another way in which monoclonal antibodies can be useful is in preparing vaccines that contain only the viral, bacterial, or parasite antigens that stimulate a protective immune response. Monoclonal antibodies can be selected that protect an animal against infection when injected (passive immunization, as described above). These antibodies can then be purified and coupled to a solid support, such as Sepharose. Such "immunoabsorbents" would then be used to purify the antigens that would then be used as vaccines. This approach eliminates the problem of obtaining killed or attenuated organisms that are uncontaminated with infective organisms, or that may be oncogenic even though no longer cytopathic, as can occur with Herpes virus. However, it does not eliminate the problem of obtaining large amounts of the microorganism in order to prepare the vaccines.

An extension of this approach involves identifying the protective monoclonal antibodies, as above, but instead of using these antibodies to purify the antigens for use as a vaccine, they are used to identify synthetic peptides that react with the monoclonal. These peptides are then used as a vaccine. Several studies suggest that such synthetic peptides can prime an animal for an immune response against the antigen recognized by the monoclonal antibody (2, 7, 34, 87, 120). Even if no antibodies are detected after immunizing with the synthetic peptide, the animal makes a secondary antibody response (antibodies appear more quickly and in larger amounts than seen with no priming) upon immunization with the virus.

Finally, the simplest use of monoclonals in vaccine production is to use them to identify the strain of organism causing an epidemic, so that the appropriate strain could be used in vaccine production (89). This approach would be especially useful for antigenically unstable viruses. Serological examination of stable viruses would also be useful when vaccination is not effective, to determine whether there has been some minor antigenic change.

CONCLUSION

Antigenic determinants of microorganisms change frequently in nature. An essential property of the immune system is its ability to protect the organism from infectious agents, no matter how genetically unstable they may be. The apparently infinite instability of antigens is therefore reflected in an enormous diversity in the amino acid sequence of antibodies. Monoclonal antibodies have made it possible to study with exquisite sensitivity the primary sequence changes that occur in naturally occuring antigens and to recreate such changes in vitro. Further, they allow the location within the three-dimensional structure of amino acid substitutions and antigenic determinants, and the determination of the frequency of mutations.

Structural analysis of the monoclonal antibodies reveals an enormous instability of immunoglobulin genes. Presumably this instability has evolved to deal with the frequent antigenic changes that occur in nature. Monoclonal anti-idiotypic antibodies have been used to analyze and manipulate the expression of this diversity.

Finally, monoclonal antibodies have provided new opportunities for the diagnosis of infectious diseases and novel approaches to inducing protective immunity.

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Literature Cited

1. Abrams, P. G., Knost, J. A., Clarke, G., Wilburn, S., Oldham, R. K. et al. 1983. *J. Immunol.* 131:1201–4
2. Arnon, R. 1980. *Ann. Rev. Microbiol.* 34:593–618
3. Avrameas, S. 1983. In *New Developments in Diagnostic Virology*, ed. P. A. Bachmann, pp. 93–99. New York: Springer-Verlag
4. Baltimore, D. 1981. *Cell* 24:592–94
5. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., et al. 1978. *Cell* 14:9–20
6. Baumal, R., Birshtein, B. K., Coffino, P., Scharff, M. D. 1973. *Science* 182:164–66
7. Beachey, E. H., Seyer, J. M., Dale, J. B., Hasty, D. L. 1983. *J. Biol. Chem.* 258:13250–57
8. Berek, C. 1983. *Eur. J. Immunol.* 13:766–72
9. Berek, C. 1984. *Ann. Immunol.* 135(C):131–36
10. Berek, C., Schreier, M. H., Sidman, C. L., Jaton, J.-C., Kocher, H., et al. 1980. *Eur. J. Immunol.* 10:258–63
11. Bernard, O., Hozumi, N., Tonegawa, S. 1978. *Cell* 15:1133–44
12. Birrer, M. J., Bloom, B. R., Udem, S. 1981. *Virology* 108:381–90
13. Bothwell, A. L. M., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., et al. 1981. *Cell* 24:625–37
14. Breschkin, A. M., Ahern, J., White, D. O. 1981. *Virology* 113:130–40
15. Bruggemann, M., Radbruch, A., Rajewsky, K. 1982. *EMBO J.* 1:629–34
16. Burstin, S. J., Spriggs, D. R., Fields, B. N. 1982. *Virology* 117:146–55
17. Caton, A. J., Brownlee, G. G., Yewdell, J. W., Gerhard, W. 1982. *Cell* 31:417–27
18. Cazenave, P.-A. 1977. *Proc. Natl. Acad. Sci. USA* 74:1522–26
19. Cerny, J., Cronkhite, R., Heusser, C. 1983. *Eur. J. Immunol.* 13:244–48
20. Charron, D. J., McDevitt, H. O. 1979. *Fed. Proc.* 38:1280a (Abstr.)
21. Cleveland, W. L., Wassermann, N. H., Sarangarajan, R., Penn, A. S., Erlanger, B. F. 1983. *Nature* 305:56–57
22. Clevinger, B., Thomas, J., Davie, J. M., Schilling, J., Bond, M., et al. 1981. In *ICN-UCLA Symposia on Cellular and Molecular Biology*, eds. C. Janeway, E. E. Sercarz, H. Wigzell, C. F. Fox, 20:159–68. New York: Academic
23. Coffino, P., Baumal, R., Laskov, R., Scharff, M. D. 1972. *J. Cell. Physiol.* 79:429–40
24. Cook, W. D., Scharff, M. D. 1977. *Proc. Natl. Acad. Sci. USA* 74:5687–91
25. Cook, W. D., Rudikoff, S., Giusti, A., Scharff, M. D. 1982. *Proc. Natl. Acad. Sci. USA* 79:1240–44
26. Crews, S., Griffin, J., Huang, H., Calame, K., Hood, L. 1981. *Cell* 25:59–66
27. Dangl, J. L., Herzenberg, L. A. 1982. *J. Immunol. Methods.* 52:1–14
28. Davies, D. R., Metzger, H. 1983. *Ann. Rev. Immunol.* 1:87–117
29. Dildrop, R., Bruggemann, M., Radbruch, A., Rajewsky, K., Beyreuther, K. 1982. *EMBO J.* 1:635–40
30. Dosseto, M., Rohner, C., Pierres, M., Goridis, C. 1981. *J. Immunol. Methods* 41:145–53
31. Dowdle, W. R., Colman, M. T., Gregg, M. 1974. *Progr. Med. Virol.* 17:91–135
32. Dwyer, D. S., Bradley, R. J., Urquhart, C. K., Kearney, J. K. 1983. *Nature* 301:611–14
33. Emini, E. A., Dorner, A. J., Dorner, L. F., Jameson, B. A., Wimmer, E. 1983. *Virology* 124:144–51
34. Emini, E. A., Jameson, B. A., Wimmer, E. 1983. *Nature* 304:699–703
35. Ertl, H. C. J., Finberg, R. W. 1984. *Proc. Natl. Acad. Sci. USA*. In press
35a. Ertl, H. C. J., Homans, E., Tournas, S., Finberg, R. W. 1984. *J. Exp. Med.* In press
36. Fazekas de St. Groth, S., Scheidegger, D. 1980. *J. Immunol. Methods* 35:1–21
37. Forstrom, J. W., Nelson, K. A., Nepom, G. T., Hellstrom, I., Hellstrom, K. E. 1983. *Nature* 303:627–29
38. Fox, P. C., Berenstein, E. H., Siraganian, R. P. 1981. *Eur. J. Immunol.* 11:431–34

39. Galfre, G., Milstein, C. 1980. *Methods in Enzymology* 73B:3–46
40. Gearhart, P. J., Johnson, N. D., Douglas, R., Hood, L. 1981. *Nature* 291:29–34
41. Gearhart, P. J., Bogenhagen, D. F. 1983. *Proc. Natl. Acad. Sci. USA* 80:3439–43
42. Gerhard, W., Webster, R. G. 1978. *J. Exp. Med.* 148:383–92
43. Goding, J. W., Burns, G. F. 1981. *J. Immunol.* 127:1256–58
44. Gough, N., Bernard, O. 1981. *Proc. Natl. Acad. Sci. USA* 78:509–13
45. Green, N., Alexander, H. H., Olsen, A., Alexander, S., Shinnick, T., et al. 1982. *Cell* 28:477–87
46. Gutman, G. A., Warner, N. L., Harris, A. W., Bowles, A. 1978. *J. Immunol. Methods* 21:101–9
47. Hayes, E. C., Lee, P. W. K., Miller, S. E., Joklik, W. K. 1981. *Virology* 108:156–63
48. Haynes, B. F., Hemler, M., Cotner, T., Mann, D. L., Eisenbarth, G. S., et al. 1981. *J. Immunol.* 127:347–51
49. Herzenberg, L. A., Herzenberg, L. A., Milstein, C. 1978. In *Handbook of Experimental Immunology*, ed. D. M. Weir, pp. 25.1–25.7. London: Blackwell
50. Honjo, T. 1983. *Ann. Rev. Immunol.* 1:499–528
51. Hunter, W. M. 1978. In *Handbook of Experimental Immunology*, ed. D. M. Weir, pp. 14.1–14.40. London: Blackwell
52. Kaartinen, M., Griffiths, G. M., Hamlyn, P. H., Markham, A. F., Karjalainen, K., et al. 1983. *J. Immunol.* 130:937–45
53. Kaartinen, M., Griffiths, G. M., Markham, A. F., Milstein, C. 1983. *Nature* 304:320–24
54. Kauffman, R. S., Noseworthy, J. H., Nepom, J. T., Finberg, R., Fields, B. N., et al. 1983. *J. Immunol.* 131:2539–41
55. Kearney, J. F., Radbruch, A., Liesegang, B., Rajewsky, K. 1979. *J. Immunol.* 123:1548–50
56. Kearney, J. F., Barletta, R., Quan, Z. S., Quintans, S. 1981. *Eur. J. Immunol.* 11:877–83
57. Kenny, P. A., McCaskill, A. C., Boyle, W. 1981. *Aust. J. Exp. Biol. Med. Sci.* 59(Pt. 4):427–37
58. Kim, S., Davis, M., Sinn, E., Patten, P., Hood, L. 1981. *Cell* 27:573–81
59. Kohler, G., Milstein, C. 1975. *Nature* 256:495–97
60. Koprowski, H., Gerhard, W., Croce, C. M. 1977. *Proc. Natl. Acad. Sci. USA* 74:2985–88
61. Koprowski, H., Steplewski, Z., Herlyn, D., Herlyn, M. 1978. *Proc. Natl. Acad. Sci. USA* 75:3405–9
62. Kung, P. C., Goldstein, G., Reinherz, E. L., Schlossman, S. F. 1979. *Science* 206:347–49
63. Kwan, S.-P., Yelton, D. E., Scharff, M. D. 1980. In *Genetic Engineering*, ed. J. K. Setlow, A. Hollander, 2:31–46. New York: Plenum
64. Lane, H. C., Butler, J. L., Fauci, A. S. 1983. In *Monoclonal Antibodies 1982 Progress and Perspectives*, ed. F. Dammacco, G. Doria, A. Pinchera, pp. 23–43. Amsterdam: Elsevier. 171 pp.
65. Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W., et al. 1979. *Virology* 98:226–37
66. Laver, W. G., Gerhard, W., Webster, R. G., Frankel, M. E., Air, G. M. 1979. *Proc. Natl. Acad. Sci. USA* 76:1425–29
67. Lee, P. W. K., Hayes, E. C., Joklik, W. K. 1981. *Virology* 108:156–63
68. Lerner, R. A. 1982. *Nature* 299:592–96
69. Levy, R., Dilley, J., Fox, R. I., Warnke, R. 1979. *Proc. Natl. Acad. Sci. USA* 76:6552–56
70. Littlefield, J. W. 1964. *Science* 145:709–10
71. Lostrom, M. E., Stone, M. R., Tam, M., Burnette, W. N., Pinter, A., et al. 1979. *Virology* 98:336–50
72. Lubeck, M. D., Schulman, J. L., Palese, P. 1980. *Virology* 102:458–62
73. Lubeck, M., Gerhard, W. 1982. *Virology* 118:1–7
74. Martinis, J., Croce, C. M. 1978. *Proc. Natl. Acad. Sci. USA* 75:2320–23
75. McGrath, M. S., Pillemer, E., Weissman, I. L. 1980. *Nature* 285:259–61
76. McMichael, A. J., Pilch, J. R., Galfre, G., Mason, D. Y., Fabre, J. W., et al. 1979. *Eur. J. Immunol.* 9:205–10
77. Milner, E. C. B., Capra, J. D. 1983. *Mol. Immunol.* 20:39–46
78. Minor, P. D., Schield, G. L., Bootman, J., Evans, D. M. A., Ferguson, M., et al. 1983. *Nature* 305:674–79
79. Moller, G., ed. 1979. *Immunol. Rev.* 47:3–252
80. Moller, G., ed. 1982. *Immunol. Rev.* 62:5–216
81. Morahan, G., Berek, C., Miller, J. F. A. P. 1983. *Nature* 301:720–22
82. Morrison, S. L., Scharff, M. D. 1981. *CRC Crit. Rev. Immunol.* 3:1–22
83. Muller, C. E., Rajewsky, K. 1983. *J. Immunol.* 131:877–81
84. Nadler, L. M., Stashenko, P., Hardy, R., Schlossman, S. F. 1980. *J. Immunol.* 125:570–77
85. Newman, B., Sugii, S., Kabat, E. A., Torii, M., Clevinger, B. L., et al. 1983. *J. Exp. Med.* 157:130–40
86. Newton, S. E., Air, G. M., Webster, R.

G., Laver, W. G. 1983. *Virology* 128:495–501
87. Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., et al. 1983. *Proc. Natl. Acad. Sci. USA* 80:4949–53
88. Nowinski, R. C., Pickering, R., O'Donnell, B. Y., Prater, A., Hammerling, U. 1981. *Virology* 111:84–92
89. Nowinski, R. C., Tam, M. R., Goldstein, L. C., Strong, L., Kuo, C.-C., et al. 1983. *Science* 219:637–44
90. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A., Herzenberg, L. A. 1978. *Cur. Top. Microbiol. Immunol.* 8:115–129
91. Oi, V., Morrison, S. L., Herzenberg, L. A., Berg, P. 1983. *Proc. Natl. Acad. Sci. USA* 80:825–29
92. Olsson, L., Kronstrom, H., Cambon-De Mouzon, A., Honsik, C., Brodin, T., et al. 1983. *J. Immunol. Methods* 61:17–32
93. Omary, M. B., Trowbridge, I. S., Minowada, J. 1980. *Nature* 286:888–91
94. Parham, P., Bodmer, W. F. 1978. *Nature* 276:397–99
95. Pawlita, M., Mushinski, E. B., Feldman, R. J., Potter, M. 1981. *J. Exp. Med.* 154:1946–56
96. Pearson, T. W., Pinder, M., Roelants, G. E., Kar, S. K., Lundin, L. B., et al. 1980. *J. Immunol. Methods* 34:141–54
97. Pech, M., Hochtl, J., Schnell, H., Zachau, H. G. 1981. *Nature* 291:668–70
98. Potocnjak, P., Yoshida, N., Nussenzweig, R. S., Nussenzweig, V. 1980. *J. Exp. Med.* 151:1504–13
99. Radbruch, A., Liesegang, B., Rajewsky, K. 1980. *Proc. Natl. Acad. Sci. USA* 77:2909–13
100. Rajewsky, K., Takemori, T. 1983. *Ann. Rev. Immunol.* 1:569–607
101. Reagan, K. J., Wunner, W. H., Wiktor, T. J., Koprowski, H. 1983. *J. Virol.* 48:660–66
102. Reinherz, E. L., Kung, P. C., Goldstein, G., Schlossman, S. F. 1979. *Proc. Natl. Acad. Sci. USA* 76:4062–66
103. Ritz, J., Pesando, J. M., Notis-McConarty, J., Lazarus, H., Schlossman, S. F. 1980. *Nature* 283:583–85
104. Rocca-Serra, J., Mazie, J.-C., Moiner, D., Leclercq, L., Somme, G., et al. 1982. *J. Immunol.* 129:2554–58
105. Rodwell, J. D., Gearhart, P. J., Karush, F. 1983. *J. Immunol.* 130:313–16
105a. Rothstein, T. L., Gefter, M. L. 1983. *Mol. Immunol.* 20:161–68
106. Rothstein, T. L., Margolies, M. M., Gefter, M. L., Marshak-Rothstein, A. 1982. *J. Exp. Med.* 157:795–800
107. Rudikoff, S., Potter, M. 1976. *Proc. Natl. Acad. Sci. USA* 73:2109–12
108. Rudikoff, S., Giusti, A. M., Cook, W. D., Scharff, M. D. 1982. *Proc. Natl. Acad. Sci. USA* 79:1979–83
109. Ruf, J., Tonnelle, C., Rocca-Serra, J., Moinier, D., Pierres, M., et al. 1983. *Proc. Natl. Acad. Sci. USA* 80:3040–44
110. Sablitzky, F., Radbruch, A., Rajewsky, K. 1982. *Immunol. Rev.* 67:59–71
111. Sacks, D. L., Esser, K. M., Sher, A. 1981. *J. Exp. Med.* 155:1108–19
112. Sacks, D. L., Sher, A. 1983. *J. Immunol.* 131:1511–15
113. Sakato, N., Eisen, H. N. 1975. *J. Exp. Med.* 141:1411–26
114. Schilling, J., Clevinger, B., Davie, J. M., Hood, L. 1980. *Nature* 283:34–50
115. Schmitz, H. E., Atassi, H., Atassi, M. Z. 1983. *Mol. Imm.* 20:719–26
116. Sela, M. 1969. *Science* 166:1365–74
117. Sela, M., Schechter, B., Schechter, I., Borek, F. 1967. *Cold Spr. Harb. Symp. Quant. Biol.* 32:537–45
118. Selsing, E., Storb, U. 1981. *Cell* 25:47–58
119. Sethi, K. K., Endo, T., Brandis, H. 1980. *J. Parasitol.* 66:192–96
120. Shinnick, T. M., Sutcliffe, J. G., Green, N., Lerner, R. A. 1983. *Ann. Rev. Microbiol.* 37:425–46
121. Shulman, M., Wilde, C. D., Kohler, G. 1978. *Nature* 276:269–70
122. Siekevitz, M., Huang, S. Y., Gefter, M. L. 1983. *Eur. J. Immunol.* 13:123–32
123. Siraganian, R. P., Fox, P. C., Berenstein, E. H. 1983. *Methods Enzymol.* 92:17–26
124. Slaughter, C. A., Capra, J. D. 1983. *J. Exp. Med.* 158:1615–34
125. Solomon, G., Schiffenbauer, J., Keiser, H. D., Diamond, B. 1983. *Proc. Natl. Acad. Sci. USA* 80:850–54
126. Spriggs, D. R., Fields, B. N. 1982. *Nature* 297:68–70
127. Stone, M. R., Nowinski, R. C. 1980. *Virology* 100:370–81
128. Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshead, J., et al. 1981. *Proc. Natl. Acad. Sci. USA* 78:4515–19
129. Teillaud, J.-L., Desaymard, C., Giusti, A. M., Haseltine, B., Pollock, R. R., et al. 1983. *Science* 222:721–26
130. Thammana, P., Scharff, M. D. 1983. *Eur. J. Immunol.* 13:614–19
131. Tonegawa, S. 1983. *Nature* 302:575–81
132. Underwood, P. A. 1981. *J. Gen. Virol.* 62:153–69
133. Urbain, J., Wikler, M., Franssen, J. O., Colligman, C. 1977. *Proc. Natl. Acad. Sci. USA* 74:5126–30
134. Van Regenmortel, M. H. V. 1984. In *Hybridoma Technology in Agriculture and Veterinary Research*, ed. N. J. Stern, in press. Totowa, NJ: Rowman & Allanheld

135. Verhoeyen, M., Faug, R., Min Jou, W., Devos, R., Huylebroek, D., et al. 1980. *Nature* 286:771–76
136. Webster, R. G., Laver, W. G. 1975. In *Influenza Viruses and Influenza*, ed. E. K. Kilbourne, pp. 170–315. New York: Academic
137. Webster, R. G., Kendal, A. P., Gerhard, W. 1979. *Virology* 96:258–64
138. Webster, R. G., Laver, W. G. 1980. *Virology* 104:139–48
139. Weigert, M. G., Cesari, I. M., Yonkovich, S., Cohn, M. 1970. *Nature* 228:1045–47
140. Wiktor, T. J., Koprowski, H. 1978. *Proc. Natl. Acad. Sci. USA* 75:3938–42
141. Wiktor, T. J., Koprowski, H. 1980. *J. Exp. Med.* 152:99–112
142. Wiley, D. C., Wilson, I. A., Skehel, J. J. 1981. *Nature* 289:373–79
143. Wilson, I. A., Skehel, J. J., Wiley, D. C. 1981. *Nature* 289:366–73
144. Winter, G., Fields, L. S., Brownlee, G. G. 1981. *Nature* 292:72–75
145. Wisdom, G. B. 1976. *Clin. Chem.* 22:1243–55
146. Yeh, M.-Y., Hellstrom, I., Brown, J. P., Warner, G. A., Hansen, J. A., et al. 1979. *Proc. Natl. Acad. Sci. USA* 76:2927–31
147. Yelton, D. E., Scharff, M. D. 1982. *J. Exp. Med.* 156:1131–48
148. Yewdell, J. W., Webster, R. G., Gerhard, W. 1979. *Nature* 279:246–48
149. Yewdell, J. W., Gerhard, W. 1981. *Ann. Rev. Microbiol.* 35:185–206
150. Yolken, R. H. 1983. In *New Developments in Diagnostic Virology*, ed. P. A. Bachmann, pp. 177–95. New York: Springer
151. Zimmerman, U. 1982. *Biochem. Biophys. Acta* 694:227–77

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BACTERIAL GLYCOGEN SYNTHESIS AND ITS REGULATION

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INTRODUCTION

Glycogen, a polysaccharide that contains only glucose with α1,4 linkages and is branched via α1,6 linkages, has been found in many bacteria as well as in eukaryotic organisms. It is generally considered to be a storage compound providing both carbon and energy for the microbes that accumulate them. In order for a compound to be classified as having energy-storage function, it was proposed (171) that it satisfy three criteria. (*a*) It must be accumulated intracellularly under conditions when the energy supply for growth of the organism is in excess. (*b*) It must be utilized when exogenous supplies of energy or carbon are no longer available for maintenance for growth or other processes necessary to sustain viability (e.g. energy needed for osmotic regulation, for maintenance of intracellular pH, for motility, for turnover of proteins and nucleic acids, or for other phenomena such as sporulation and encystment). The energy needed for the latter processes, which are not necessarily involved in cell division and growth, has been defined as energy of maintenance. (*c*) Perhaps the most important criterion is that the proposed storage compound is degraded to an energy form that is utilized by the cell, enabling it to fare or survive better in the environment.

The studies indicating that glycogen fits the above criteria will be briefly summarized. However, the main objective of this review is to present the information presently known about the enzymatic processes involved in bacterial glycogen systems and the associated allosteric and genetic regulatory phenomena.

Glycogen synthesis usually occurs at periods of slow growth or no growth. Thus a study of the modulation of the synthesis of the polymer may provide some insight into the regulatory modes that appear in the stationary phases of growth of the bacterium. Previous reviews (33, 75, 113–116, 119, 130) on bacterial glycogen have appeared and some will be referred to with respect to the material discussed here. Space does not permit a description of the enzymatic properties of the bacterial glycogen synthase and branching enzyme. However, these enzymes have recently been reviewed (119) and their various properties reported (68).

PHYSIOLOGICAL CONDITIONS CAUSING GLYCOGEN ACCUMULATION

Accumulation of glycogen usually occurs as a result of limited growth conditions in the presence of an excess source of carbon. Many bacterial species accumulate glycogen in the stationary phase, when growth ceases because of either depletion of a nutrient such as nitrogen (66, 67, 90, 98, 145, 153, 160, 178), sulfur (145, 178), or phosphate (178), or even because of an unfavorable

pH for growth (178). Holme in 1957 (66) showed that in *E. coli* B the rate of growth and the quantity of glycogen accumulated had an inverse relationship when the cells were grown in cultures having glucose as the carbon source and limited with respect to nitrogen. Exceptions to this, where glycogen does accumulate to optimal levels during the exponential phase of growth, have been observed with cultures of *Streptococcus mitis* (48) and *Rhodopseudomonas capsulata* (41). Glycogen, however, does not appear to be necessary for bacterial growth; many mutants of *E. coli* (17, 49, 50, 125), *Salmonella typhimurium* (158), and *Clostridium pasteurianum* (32), deficient in glycogen synthesis, as well as a mutant of *E. coli* having a deletion of the structural genes of the glycogen biosynthetic enzymes (29, 144), grow as well as their normal parent strains.

In a previous review (119) it was indicated that glycogen-like reserves had been reported in 29 different bacterial species. This list has grown in the last three years and includes bacteria like *Acetivibrio cellulolyticus* (109), *Streptomyces viridochromogenes* (7), *Streptomyces antibioticus, Streptomyces fluorescens, Streptomyces griseus* (8), and *Selenomonas ruminantium* (71); the Thermoacidophilic archaebacteria of the genera *Sulfolobus, Thermoproteus, Desulfurococcus,* and *Thermococcus* (74); a species of the cyanobacterium *Anabaena* (142); fast-growing species of *Rhizobium* (179), *R. leguminosarium, R. phaseoli, R. trifolii,* and *R. meliloti*; *Nocardia asteroides* (42) and *Aeromonas hydrophila* (146).

It was found that the greatest amount of glycogen accumulation in the organisms mentioned above occurred at nitrogen-limiting conditions. However, optimal glycogen accumulation was observed with the archaebacterial strain *Sulfolobus solfataricus* (74) only under optimal growth conditions. At the onset of sporogenesis in *S. viridochromogenes,* a glycogen-like polymer accumulated (7); this glycogen accumulation during sporogenesis followed a pattern similar to that described in other sporulating microorganisms such as *Clostridia* strains (89, 161, 162).

In a species of *Anabaena,* glycogen accumulation is a light-dependent process (142). Large quantities are accumulated in cells undergoing sporulation. Glycogen did not accumulate in the dark or in the presence of DCMU in the light. Because DCMU is an inhibitor of photophosphorylation, this would suggest that glycogen is accumulated under conditions of "energy excess."

BIOLOGICAL FUNCTION OF BACTERIAL GLYCOGEN

E. coli (159), *Enterobacter aerogenes* (160), and *Streptococcus mitis* (168) glycogen-containing cells in media with no exogenous carbon source have prolonged viability compared to their glycogen-less counterparts under starvation conditions. It was also observed that, under starvation conditions, *E. coli*

and *E. aerogenes* (133, 159) that contain glycogen do not degrade their RNA and protein components to NH_3, in contrast to those cells having no glycogen. Thus, it is believed that the presence of glycogen preserves some cellular constituents that are susceptible to turnover in stationary phase. It should be pointed out, however, that in media containing 0.5–1.0 mM $MgCl_2$, both glycogen-containing and glycogen-deficient cells had the same survival rate (159, 165). Nevertheless, this observation does not rule out the role of glycogen as an energy reserve in conditions with low levels of Mg^{2+} or other physiological situations. $MgCl_2$ apparently increases the stability of the ribosomes, the major RNA and protein components in the cell, thus decreasing their turnover rates and lowering the energy requirement of maintenance (91, 92, 95, 96, 111). The resynthesis of proteins in *E. coli* during turnover probably involves the major portion of the maintenance energy (92).

For various *Clostridia* species the α1,4 glucan counterpart, granulose, may accumulate up to 60% of their dry weight prior to the onset of sporulation (89, 161, 162). The accumulated glucan is rapidly degraded with concomitant spore formation, and this suggests that the polysaccharide serves as a source of energy and carbon for formation of spores and their maturation. This may be also the case for other spore-forming bacteria. As previously mentioned, glycogen granules appeared in sporulating hyphae of *Streptomyces viridochromogenes* and reached a maximum number during early stages of maturation (7). During later stages of maturation the number of granules decreased, and no granules were seen in mature spores. Glycogen was also shown to accumulate in *Bacillus cereus* early in sporulation and degraded at the time of spore maturation (154).

Thus, in sporulating microorganisms, it has been suggested that glycogen or granulose may serve as an endogenous source of carbon and energy for spore maturation.

In summary, a number of experiments suggest that glycogen may play a role in the survival of the bacterial cell. The precise function of glycogen in this role, however, remains unclear. However, a number of studies seem to be in conflict with glycogen as an energy-storage compound. As indicated above, the survival rates of magnesium-rich *E. coli* and *E. aerogenes* cells are not affected whether they contain glycogen or not (159, 165). Moreover, glycogen-rich *Sarcina lutea* dies at a faster rate than cells containing no polysaccharide when starved in phosphate buffer (10). It is clear that other studies must be done to clarify the function of glycogen in bacteria.

It should also be pointed out that the synthesis and subsequent degradation of glycogen by oral bacteria such as *Streptococcus mutans* (69) or *S. mitis* (48) may be an important factor in the development of dental caries (164, 169). Consistent with the suggestion is the finding that organisms capable of synthesizing glycogen produce more acid when exogenous carbohydrate is present and can produce acid in the absence of exogenous carbohydrate, as compared to those bacteria unable to synthesize glycogen (69).

The acid formed from polysaccharide catabolism may be of significance in production of dental caries, for it is formed over a considerable period of time and consequently may be responsible for the lower resting pH that has been observed in plaque from individuals with active caries (69).

ENZYMATIC SYNTHESIS OF BACTERIAL GLYCOGEN

Synthesis of glucosyl linkages in either polysaccharides or various glycoconjugates is usually mediated by transfer of the glucosyl unit from a sugar nucleotide to an appropriate acceptor. This is also true for glycogen synthesis in bacteria. The preponderant evidence indicates that the glycosyl donor is adenosine diphosphate glucose (ADP-glucose). ADP-glucose is made via a reaction (Reaction 1) of ATP with α-glucose-1-P catalyzed by the enzyme, ADP-glucose pyrophosphorylase (ATP:α-glucose-1-P adenylyltransferase, E. C. 2.7.7.27) (113, 115, 116, 119, 148).

$$\text{ATP} + \alpha\text{-glucose-1-P} \rightleftharpoons \text{ADP-glucose} + PP_i \qquad 1.$$

A transfer of the glucosyl unit from ADP-glucose to a preexisting α1,4 glucan or maltodextrin primer to form a new α1,4 glucosyl linkage is catalyzed by an ADP-glucose-specific glycogen synthase (ADP-glucose:1,4 α-D-glucan 4-α glucosyl transferase, E. C. 2.4.1.21; Reaction 2) (45, 51, 149).

$$\text{ADP-glucose} + \alpha 1,4 \text{ glucan} \rightarrow \alpha 1,4 \text{ glucosyl-glucan} + \text{ADP} \qquad 2.$$

The α1,6 glucosyl linkages, which are about 10% of the total linkages found in glycogen, are formed in a reaction catalyzed by branching enzyme (α1,4 glucan:α1,4 glucan-6 glycosyltransferase, E. C. 2.4.1.18). Branching-enzyme activity has been detected in enteric bacteria (6, 68, 120, 152, 158), *Arthrobacter globiformis* (177), and in some photosynthetic bacteria (52, 53, 122).

It should be pointed out, however, that some α-glucans similar to glycogen or amylopectin in structure can be formed either directly from sucrose or from maltose in reactions catalyzed by amylosucrase (Reaction 3) and amylomaltase (Reaction 4).

$$\text{Sucrose} + (1,4\text{-}\alpha\text{-D-glucosyl})_n \rightleftharpoons \text{D-fructose} + (1,4\text{-}\alpha\text{-D-glucosyl})_{n+1} \qquad 3.$$

$$\text{Maltose} + (1,4\text{-}\alpha\text{-D-glucosyl})_n \rightleftharpoons \text{glucose} + (1,4\text{-}\alpha\text{-D-glucosyl})_{n+1} \qquad 4.$$

Various *Neisseria* strains, when grown on sucrose (60–63, 65, 88, 102), accumulate large amounts of α1,4 glucan and contain substantial amounts of amylosucrase. It appears, however, that amylosucrase is present in only a few bacterial species and is active only if sucrose is present as a carbon source. Sucrose has never been reported to be synthesized by either *Neisseria* or any other microorganism. Thus the role of amylosucrase in the synthesis of α1,4 glucans in bacteria appears to be limited.

Amylomaltase, the enzyme that catalyzes Reaction 4, is induced along with a number of other enzymes when various strains of *Aerobacter aerogenes* (106), *Diplococcus pneumoniae* (77), *E. coli* (106), *Streptococcus mitis* (170), *Streptococcus mutans* (106), *Streptococcus pyogenes* (94), and *Pseudomonas stutzeri* (172) are grown on maltose or maltodextrins. Synthesis of amylomaltase is repressed when glucose is in the media (19) and thus is an inoperative enzyme when glycogen is accumulated in organisms grown on glucose as a carbon source.

α1,4 Glucosyl linkages could also be formed via reactions catalyzed by maltodextrin phosphorylase and glycogen phosphorylase (Reaction 5); these enzymes are found in a number of bacterial extracts (65, 119).

$$\alpha 1,4\text{-(glucosyl)}_{n+1} + P_i \rightleftharpoons \alpha\text{-glucose-1-P} + \alpha 1,4\text{-(glucosyl)}_n \qquad 5.$$

The maltodextrin phosphorylase, however, is only induced in the presence of maltose or maltodextrins and is an enzyme whose function is to degrade maltodextrins to glucose-1-P. This is supported by the observation that *E. coli* mutants that are deficient in maltodextrin phosphorylase accumulate maltodextrins (143). The activity of glycogen phosphorylase found in many bacterial extracts is usually insufficient to account for the observed rate of glycogen accumulation (20, 21, 73).

It appears that most, if not all, bacteria accumulate glycogen via the ADP-glucose pathway. Either glycogen-deficient or glycogen-hyperproducing mutants of *E. coli* (17, 31, 49, 50, 75, 113–116, 119, 121, 123, 125, 128) and of *S. typhimurium* (158) are affected in glycogen-synthase activity, in ADP-glucose–pyrophosphorylase activity, or in both. A 1981 review (119) listed in a table 42 different bacterial strains whose extracts contained substantial levels of both ADP-glucose pyrophosphorylase and glycogen synthase. Subsequent to that review, three studies (51, 53, 176) have appeared that have increased the number of bacterial strains containing the glycogen biosynthetic enzymes by four *(Aeromonas liquefaciens, Edwardsiella tarda, Enterobacter hafniae* and *Rhodopseudmonas blastica)* as well as describing in some detail the properties of the ADP-glucose pyrophosphorylases from related bacteria.

ALLOSTERIC REGULATION OF ADP-GLUCOSE–PYROPHOSPHORYLASE ACTIVITY

Activators

For most bacterial systems tested, it has been found that glycolytic intermediates are activators for ADP-glucose synthesis. The presence of glycolytic intermediates are required for optimal activity, and they may be considered as indicators of carbon in excess of energy needs and thus can be used for production of an energy-storage compound.

The finding that regulation occurs at the ADP-glucose–synthesis step is consistent with the concept that regulation of a biosynthetic pathway occurs at the first unique step of the pathway.

Table 1 lists the activator specificity of the ADP-glucose pyrophosphorylases studied. Seven groups can be formulated based on the differences in the activator specificity observed. It is also seen in Table 1 that the various groups may have an overlapping specificity with respect to activators. The most prevalent activators are fructose 6-P, fructose 1,6-P_2, 3-phosphoglycerate, and pyruvate. Furthermore, fructose 6-P as well as fructose 1,6-P_2 can activate the cyanobacterial ADP-glucose pyrophosphorylase, but not as well as 3-P-glycerate (86). 3-P-glycerate can also activate to a slight extent the enteric bacterial ADP-glucose pyrophosphorylase, but certainly not to the extent observed for fructose 1,6-P_2 (129). This overlapping specificity of activator observed in the various classes suggests that the activator sites for the different groups may be related. It is quite possible that a portion of the gene coding for the activator site of the ADP-glucose pyrophosphorylase was modified during evolution, with the pressure being the coordination of the activation phenomena with the carbon assimilatory pathway prevalent in the organism.

Previous reviews (113, 115, 116, 119) have discussed to a great extent the congruency of activator specificity with the various carbon assimilatory pathways present in the bacteria. For example, it appears that when an organism obtains its energy via the glycolytic pathway, its ADP-glucose pyrophosphorylase would be activated by fructose 1,6-P_2. On the other hand, the cyanobacteria that assimilate carbon via the ribulose bisphosphate carboxylase pathway contain ADP-glucose pyrophosphorylases activated by the CO_2-fixation product, 3-P-glycerate. Generally the activators tend to increase the apparent affinity of the substrates ATP and glucose 1-P for the enzyme (113, 115, 116).

Because a number of the ADP-glucose pyrophosphorylases are activated by fructose 1,6-P_2, it was of interest to know if fructose 2,6-P_2, a newly discovered metabolite in mammalian and plant tissues, (30, 64, 97, 110, 140, 141, 167) and shown to be an activator of liver and plant phosphofructokinases and inhibitor for the liver and plant fructose 1,6-bis-phosphatases, was also an

Table 1 Classes of bacterial ADP-glucose pyrophosphorylases based on allosteric activator specificity

Activator(s)	Bacteria
Pyruvate	*Rhodospirillum* sp.; *R. fulvum, R. molischianum, R. photometricum, R. rubrum, R. tenue; Rhodocyclus purpureus*
3-P-glycerate	*Aphanocapsa* 6308, *Synechococcus* 6301
Pyruvate Fructose 6-P	*Agrobacterium tumefaciens, Arthrobacter viscosus, Chlorobium limicola, Chromatium vinosum, Rhodopseudomonas* sp.; *R. acidophila, R. blastica, R. capsulata, R. palustris*
Fructose 6-P Fructose 1,6-P_2	*Aeromonas hydrophila, Micrococcus luteus, Mycobacterium smegmatis, Rhodopseudomonas viridis*
Pyruvate Fructose 6-P Fructose 1,6-P_2	*Rhodopseudomonas* sp.; *R. gelatinosa, R. globiformis, R. sphaeroides*
Fructose 1,6-P_2 Pyridoxal-P NADH	*Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter cloacae, Escherichia aurescens, Escherichia coli, Klebsiella pneumoniae, Salmonella enteriditis, Salmonella typhimurium, Shigella dysenteriae*
None	*Clostridium pasteurianum, Enterobacter hafniae, Serratia* sp.; *S. liquifaciens, S. marcescens*

effector molecule of the bacterial or plant ADP-glucose pyrophosphorylases. Fructose 2,6-P_2 may also be considered an analogue of fructose 1,6-P_2, and fructose 1,6-P_2 has been shown to activate a number of ADP-glucose pyrophosphorylases (Table 1). The concentration of fructose 2,6-P_2 observed in the eukaryotic cells rarely exceeds 20 μM.

It was found that fructose 2,6-P_2 at 50 μM did not stimulate ADP-glucose synthesis catalyzed by either pure or partially purified ADP-glucose pyrophosphorylases from *R. blastica, R. molischianum, S. typhimurium, R. rubrum, E. hafniae, R. globiformis, E. tarda, S. dysenteriae,* spinach leaf, or potato tuber over that seen by reaction mixtures containing no activator (E. Greenberg & J. Preiss, unpublished results). Indeed, no activation of the spinach leaf enzyme was observed up to a concentration of 2.5 mM. Some activation of ADP-glucose synthesis was seen with the *E. coli* and *R. sphaeroides* enzymes, and this is shown in Figure 1. Fructose 2,6-P_2, however, is not as effective in stimulating ADP-glucose synthesis as is fructose 1,6-P_2 or fructose 6-P. Fructose 2,6-P_2 stimulates the *E. coli*–enzyme activity to the same extent as fructose 1,6-P_2, but 50% activation by fructose 2,6-P_2 is observed at 350 μM, while only about 68 μM fructose 1,6-P_2 is required (49, 56, 125). Thus, about five times more fructose 2,6-P_2 is required for activation. The same is observed with the *R. sphaeroides* enzyme with respect to half maximal–activation concentration. That value is about 9 μM for fructose 6-P (122), 15 μM for fructose 1,6-P_2 (122), and 60 μM for fructose 2,6-P_2. Furthermore, the extent of

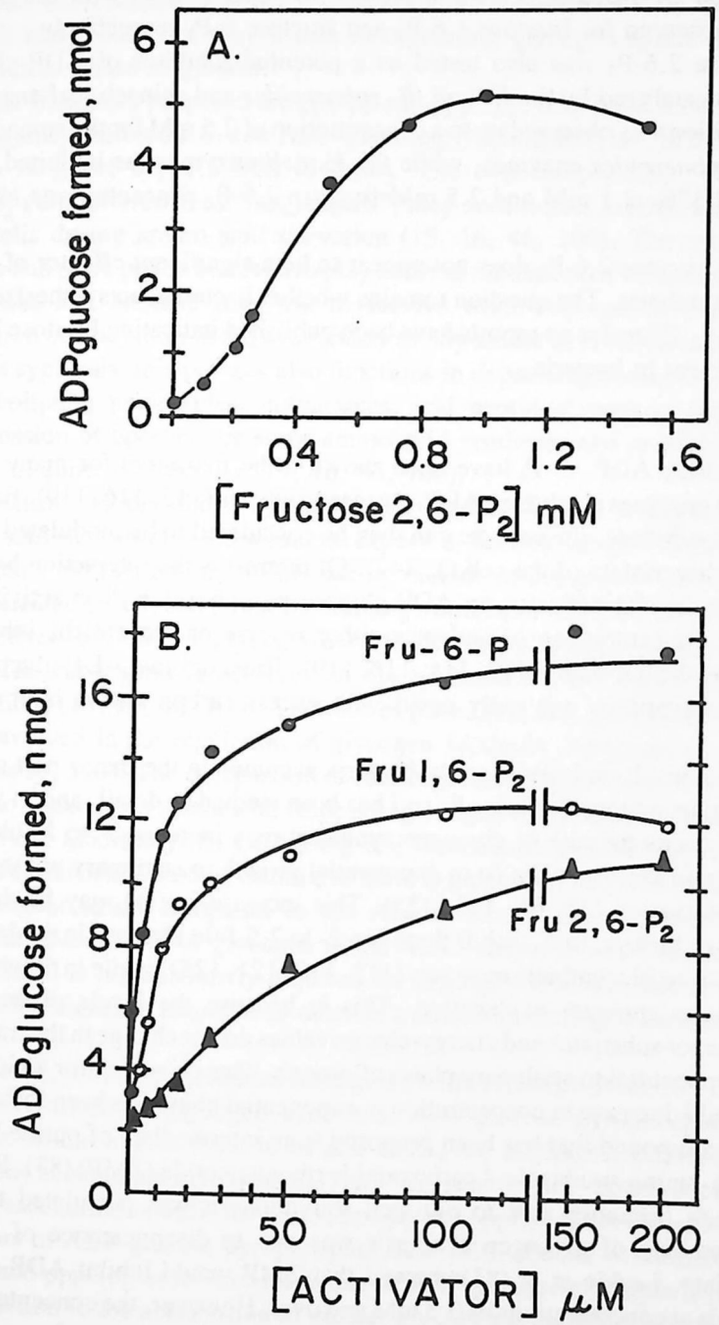


Figure 1 The activation of the *E. coli* (*A*) and *R. sphaeroides* (*B*) ADP-glucose pyrophosphorylases by fructose 2,6-P_2.

stimulation by fructose 2,6-P_2 is only 5.5-fold compared with the 7- and 10-fold observed for fructose 1,6-P_2 and fructose 6-P, respectively.

Fructose 2,6-P_2 was also tested as a potential inhibitor of ADP-glucose synthesis catalyzed by the *E. coli*, *R. sphaeroides* and spinach leaf enzymes. No inhibition was observed up to a concentration of 2.5 mM for the spinach leaf and *R. sphaeroides* enzymes, while the *E. coli* enzyme was inhibited about 11% and 33% at 1 mM and 2.5 mM fructose 2,6-P_2 concentrations, respectively.

Thus, fructose 2,6-P_2 does not appear to be a significant effector of ADP-glucose synthesis. The question remains whether bacteria can synthesize fructose 2,6-P_2. Thus far no reports have been published indicating fructose 2,6-P_2 to be present in bacteria.

Inhibitors

Either AMP, ADP, or P_i have been shown to be inhibitors for many of the bacterial enzymes catalyzing ADP-glucose synthesis (115, 116, 119). Because ATP is a substrate, the enzyme can then be considered to be modulated by the energy-charge state of the cell (1, 147). Of interest is the interaction between the inhibitor and activator on ADP-glucose pyrophosphorylase activity. Increasing concentrations of activator either reverse or prevent the inhibition caused by the inhibitors (113, 115, 116, 119). Thus, optimal ADP-glucose and glycogen synthesis may only occur with excess carbon and at high energy charge.

As previously indicated, many bacteria accumulate the major part of their glycogen in stationary phase. *E. coli* has been studied in detail, and in various *E. coli* strains the rate of glycogen synthesis may increase 2- to 5-fold upon transition of the organism from exponential growth to stationary phase due to NH_4^+ starvation (35, 66, 123, 125). This increase in rate may be due to a number of factors. In *E. coli* B there is a 2- to 2.5-fold increase in the levels of the glycogen biosynthetic enzymes (113, 119, 121, 125), while in other strains there is no apparent explanation. This is because the levels of enzymes, activator, or substrates and energy-charge values do not change in the transition from exponential to stationary phase of growth. Thus, a search for an inhibitor that would decrease in concentration in exponential phase has been looked for.

One compound that has been proposed is an intermediate of purine biosynthesis, 5-amino-imidazole-4 carboxamide ribonucleotide (ZMP) (83). Because of growth limitation due to nitrogen starvation, it was postulated that the increased rate of glycogen synthesis was due to disappearance of this intermediate. Leckie et al (83) showed that ZMP would inhibit ADP-glucose synthesis at concentrations of 0.5 mM and over. However, the concentration of ZMP has been found to be about 0.1 mM in exponential phase of growth in *S. typhimurium* Lt-2 (4) and at this concentration does not inhibit ADP-glucose synthesis (83). Most probably the concentration of ZMP would be the same in *E. coli* and would not play a role in regulating glycogen synthesis during exponential phase of growth.

Other inhibitors proposed are ppGpp and pppGpp; they have been shown to significantly inhibit the *E. coli* ADP-glucose pyrophosphorylase in the concentration range of 0.5–1.5 mM (34, 38). The two compounds, ppGpp and pppGpp, are referred to as "magic spots"; they accumulate in large amounts in rel^+ cells during amino acid starvation (15, 16, 46, 100). The nucleotides ppGpp and pppGpp are believed to play a role as mediators of stringent control, a process that bacteria may use to survive when exposed to amino acid starvation or limitation in the availability of any amino acyl-t-RNA species for protein synthesis; this process also functions in depressing synthesis of RNA, phospholipids, nucleotides, polyamines, and peptidoglycans as well as in derepression of operons for some amino acid syntheses and in depression of uptake of some metabolites (15, 16, 46, 100).

In contrast to the above syntheses, glycogen synthesis is augmented by amino acid starvation. This would be expected, because, as previously stated, glycogen synthesis mainly occurs during periods of nongrowth. Two reports also indicated that *E. coli* strains containing a defective *rel*A gene were not able to effectively accumulate glycogen as well as the *rel*A^+ gene isogenic strain (9, 163). The *rel*A gene is involved in the synthesis of the above guanosine nucleotides (15, 16, 46, 100). Thus it was postulated that ppGpp and pppGpp were involved in the regulation of glycogen synthesis during the restringent response (9, 163). The comparison of four different stringent strains with their isogenic relaxed pair strain with respect to glycogen biosynthesis has also been done in our laboratory (E. Greenberg & J. Preiss, unpublished experiments) and has confirmed the observation that there is greater synthesis of glycogen in the stringent strain compared to the relaxed strain. Because there was a significant basal level of glycogen in the *rel*A^- strain, it was concluded that *rel*A control is not absolutely required for glycogen synthesis but is rather a modulation signal in response to nutrient availability (nitrogen limitation) (9, 163).

However, if those nucleotides were going to be effectors of the rate-limiting enzyme involved in glycogen synthesis, ADP-glucose pyrophosphorylase, then one would expect them to be activators, not inhibitors, because *rel*A^+ organisms accumulate glycogen and *rel*A^- isogenic strains do not accumulate the polysaccharide as well. However, ppGpp and pppGpp were shown not to be activators of ADP-glucose pyrophosphorylase (49), and the demonstration of ppGpp and pppGpp as in vitro inhibitors of ADP-glucose synthesis is in direct contradiction to the observation of greater glycogen accumulation in the stringent strains (9, 163). In a later study, Leckie et al (84) observed that glycogen accumulation in *E. coli* *rel*A^+ or *rel*A^- isogenic strains was about the

same when glycerol was the carbon source; they concluded that ppGpp does not play a role in glycogen synthesis. The in vivo effect of ppGpp and pppGpp on glycogen accumulation may be indirect or on the induction of some enzymes that are either involved in or affect glycogen synthesis. Obviously, further studies are needed to clarify the situation.

Another potential inhibitor of ADP-glucose and glycogen synthesis may be inorganic pyrophosphate, the other product of the ADP-glucose-pyrophosphorylase reaction. It was previously thought that inorganic pyrophosphate, formed in various biosynthetic reactions, was cleaved by inorganic pyrophosphatase and thus was present in the cell in negligible concentrations. However, recent results indicate that PP_i is a normal metabolite in microorganisms (5, 43, 76). In *E. coli* it has been estimated to be at a level of about 0.5 mM during logarithmic growth in minimal media (76). It was therefore of interest to see if PP_i could affect ADP-glucose synthesis. Recently published data suggest that PP_i may play a role in the regulation of bacterial glycogen synthesis via inhibition of ADP-glucose pyrophosphorylase activity (117). ATP or the physiological activator, fructose 1,6-P_2, do not seem to affect the inhibition caused by PP_i. The inhibition is additive over AMP inhibition, and thus PP_i is probably binding to a site other than the AMP binding site. The only metabolite capable of partially reversing the inhibition to some extent would be the substrate glucose 1-P; i.e. mixed type inhibition was observed with glucose 1-P (117). The K_i was determined to be 40 μM, and thus the in vivo pyrophosphate concentration observed in exponential phase of growth is about 10-fold greater than the K_i value.

It is therefore quite possible that the increased rate of glycogen synthesis that occurs in periods of nongrowth may in part be due to a decrease in the level of the inhibitor, PP_i. During the exponential phase of growth the various biosynthetic reactions in the synthesis of various cellular constituents and macromolecules would be optimal; thus the levels of PP_i in the cells would be relatively high, because PP_i is a by-product of these biosynthetic reactions. When cellular growth ceases, as in stationary phase, then the rate of production of PP_i due to biosynthesis would decrease, and the PP_i levels may decrease because inorganic pyrophosphatase action is able to hydrolyze the lesser amounts of PP_i produced to a lower level. If this does occur, then inhibition of ADP-glucose and glycogen synthesis would be relieved.

Thus, PP_i may coordinate glycogen synthesis with cell growth. High growth rates may increase PP_i levels and thus inhibit glycogen synthesis. At lower growth rates the PP_i levels may be lower and glycogen synthetic rates may increase. Because ppGpp and pppGpp inhibit many biosynthetic processes, it is quite possible that they also indirectly mediate the lowering of PP_i concentration in the cell. This may be the basis for stimulation of glycogen synthesis in the stringent response. It would therefore be of interest to determine the levels

of PP_i in exponential and in stationary phase of growth in nitrogen-limiting conditions to determine whether there is a relationship between PP_i and rates of glycogen synthesis.

Recent results have also indicated that the purified potato tuber, spinach leaf, *R. sphaeroides*, and *R. rubrum* ADP-glucose pyrophosphorylases are also inhibited by PP_i. Figure 2 shows the effect of PP_i on ADP-glucose synthesis catalyzed by the *R. sphaeroides*, *R. rubrum*, and spinach leaf enzymes. Fifty percent inhibition can occur from about 0.05 to 0.3 mM PP_i. The sensitivity of the spinach leaf enzyme to PP_i inhibition was also affected by the concentration of glucose 1-P. Higher concentrations made the enzyme less sensitive to PP_i inhibition (Figure 2).

PP_i may also regulate many other biosynthetic reactions (78) and possibly could be visualized as a coordinating metabolite of a number of biosynthetic pathways. It also may well coordinate the various biosynthetic pathways with growth rate. PP_i has also been recognized as a substrate for a number of

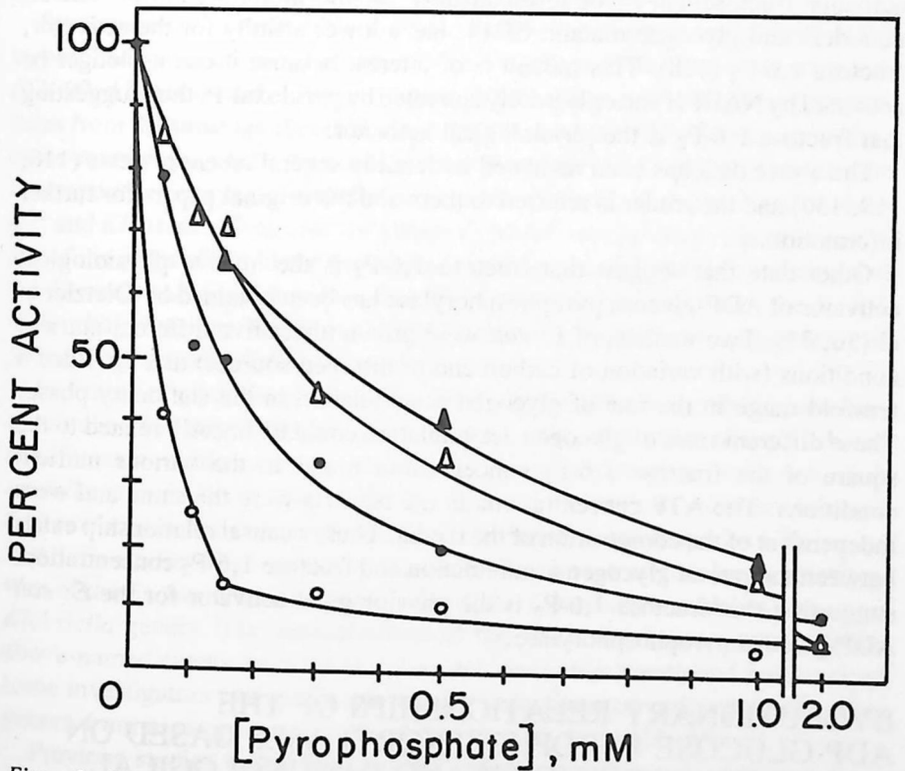


Figure 2 Pyrophosphate inhibition of ADP-glucose synthesis catalyzed by the *R. sphaeroides* (▲), *R. rubrum* (Δ), and spinach leaf (○, ●) ADP-glucose pyrophosphorylases. The concentration of glucose 1-P was 0.5 mM except for the curve with the open circles where the concentration was 0.05 mM.

reactions in microorganisms (101, 131, 132) as well as in plants (14, 30). Its function in metabolism as a substrate as well as a regulatory constituent may well be more extensive than previously thought.

Evidence Indicating That Allosteric Regulation of Bacterial ADP-glucose Pyrophosphorylase Has In Vivo Functionality

There is much evidence to show that the activation and inhibition phenomena observed in vitro are also functional in vivo in both plants and in bacteria. The evidence for the allosteric activation and inhibition being important in the regulation of bacterial glycogen synthesis has been mainly derived from a study of a class of mutants of *E. coli* B (49, 50, 113, 115, 116, 119, 121, 125), *E. coli* K12 (17, 29), and *Salmonella typhimurium* LT-2 (158) that are affected in their ability to accumulate glycogen and contain ADP-glucose pyrophosphorylases that have modified allosteric properties. The mutants accumulating glycogen at a faster rate than the parent strain either have higher apparent affinity for the activator fructose 1,6-P_2 or lower affinity for the inhibitor, AMP. The *E. coli*–deficient glycogen mutant, SG14, has a lower affinity for the activator, fructose 1,6-P_2 (128). This mutant is of interest because it can no longer be activated by NADPH and only poorly activated by pyridoxal-P, thus suggesting that fructose 1,6-P_2 is the physiological activator.

The above data has been reviewed in detail in several recent reviews (116, 119, 130) and the reader is referred to them and the original papers for further information.

Other data that suggest that fructose 1,6-P_2 is the in vivo physiological activator of ADP-glucose pyrophosphorylase has been obtained by Dietzler et al (36, 37). Two mutants of *E. coli* were grown under five different nutrient conditions (with variation of carbon and/or nitrogen sources) that provided a ten-fold range in the rate of glycogen accumulation in the stationary phase. These different rates of glycogen accumulation could be linearly related to the square of the fructose 1,6-P_2 concentration found in the various nutrient conditions. The ATP concentrations in the bacteria were the same and were independent of the composition of the media. Thus, a causal relationship exists between the rate of glycogen accumulation and fructose 1,6-P_2 concentration, suggesting that fructose 1,6-P_2 is the physiological activator for the *E. coli* ADP-glucose pyrophosphorylase.

EVOLUTIONARY RELATIONSHIPS OF THE ADP-GLUCOSE PYROPHOSPHORYLASES BASED ON ACTIVATOR SPECIFICITY AND IMMUNOLOGICAL RELATIONSHIPS

Table 1 may also suggest a rough relationship between the activator specificity between a bacterium's ADP-glucose pyrophosphorylase and its taxonomic classification. These relationships are seen for a number of related genera and classes.

Most enteric organisms accumulating glycogen have an ADP-glucose pyrophosphorylase activated by fructose-P_2, NADPH and pyridoxal-P and inhibited by AMP. Two exceptions are found, however, and they are the enzymes present in the genus *Serratia* (120) and in *Enterobacter hafniae* (176). These enzymes were not activated to any great extent by the above three compounds and appear not to be activated by any physiological compound. However, their enzymes are very sensitive to AMP inhibition and, in this respect, similar to the other enteric ADP-glucose pyrophosphorylases.

The finding of an ADP-glucose pyrophosphorylase in *Serratia* and in *E. hafniae* with allosteric properties different from those of the enzyme found in *Escherichia, Shigella, Salmonella, Citrobacter,* and *Klebsiella/Enterobacter* is consistent with previous reports of other dissimilarities of the genus *Serratia* and of *E. hafniae* to the other enteric genera, with respect to in vitro DNA hybridization studies and to other comparative protein studies.

Studies on the immunological cross-reactivities of the α and β subunits of tryptophan synthase from species of various genera of the *Enterobacteriaceae* (27, 99, 138) and on the immunological relationships of the alkaline phosphatases from the same species (22) show that the enzymes from *Serratia* have less cross-reactivity with the antiserum prepared against the *E. coli* protein than the corresponding enzymes from the *Escherichia, Shigella, Salmonella, Citrobacter,* and *Klebsiella/Enterobacter* group. Furthermore, primary sequence analyses of the alpha chain of the tryptophan synthase, partial and full, indicate that the *Serratia marcescens* protein is more dissimilar in sequence to the *E. coli* alpha chain than are the *Salmonella typhimurium, Enterbacter aerogenes,* and *Shigella dysenteria* alpha chains (27).

In a study of other enzyme activities in the tryptophan biosynthetic pathway, it was observed that the anthranilate synthase and anthranilate phosphoribosyl transferase activities are in a single large enzyme complex in *E. coli, Salmonella* sp., and *E. aerogenes* (27, 79). In *S. marcescens, S. marinorubra,* and *S. liquefaciens,* the two activities reside in separate polypeptides (79).

All the studies cited clearly demonstrate the close macromolecular similarities of the *Escherichia, Shigella, Salmonella, Citrobacter,* and *Enterobacter/Klebsiella* genera. The dissimilarities of the genus *Serratia* to genera of the above-named enteric bacteria in the numerous studies mentioned have caused some investigators to hypothesize that *Serratia* diverged earlier than the other genera from a common ancestor of the enterobacteria (22, 79).

Previous studies had shown that *E. hafniae* is sufficiently different from other *Enterobacteriaceae* so that it should be reclassified as *Hafniae alvei* (3, 79, 157). The *E. hafniae* ADP-glucose pyrophosphorylase is not activated by the metabolites that activate the other enteric enzymes (176), particularly those seen for *Enterobacter aerogenes* and *Enterobacter cloacae* (134, 176). This

difference in activator specificity would be consistent with the classification results, indicating that *E. hafniae* is indeed different from those in the genus *Enterobacter*.

Aeromonads ferment various carbohydrates to yield end products similar to those found in carbohydrate dissimilation by enterics (26, 156), and several species of *Aeromonas* contain a β-galactosidase similar to *Escherichia coli* (139). The genetic regulation and the nature of enzymes involved in tryptophan biosynthesis in *A. formicans* are very similar to those found in *E. coli* and *Salmonella typhimurium* (28). Moreover, the allosteric patterns of control of aspartokinase of *A. formicans* and *A. hydrophila* (23) and of 3-deoxy-D-arabino-heptulosonate-7-P synthetase of *A. formicans* (70) were very similar to those found with the respective enzymes of the *Enterobacteriaceae*. The 16S ribosomal RNA sequence studies of Fox et al (44), however, show that *Aeromonas* is a distinct lineage, somewhat separate from the *Enterobacteriaceae*.

Glycogen, a storage compound found in many enteric organisms, is also found along with the glycogen biosynthetic enzymes in the aeromonads (116, 146). Because of this, it was of interest to compare the allosteric properties of the *A. hydrophila* ADP-glucose pyrophosphorylase with the enteric enzymes (176).

The *A. hydrophila* ADP-glucose–pyrophosphorylase regulatory properties are quite unlike that observed for the *Enterobacteriaceae,* even though the enzyme is activated by fructose 1,6-P_2. None of the enteric ADP-glucose pyrophosphorylases have been found to be activated by fructose 6-P. Yet fructose 6-P is more potent than fructose 1,6-P_2 as an activator for the *A. hydrophila* enzyme. AMP, a very potent inhibitor of the enteric ADP-glucose pyrophosphorylases, as well as P_i, do not inhibit the *A. hydrophila* enzyme. ADP only inhibits at high concentration.

In summary, although previous studies had indicated that allosteric and genetic patterns of control and nature of some enzymes involved in amino acid biosynthesis in aeromonads were similar to that observed in the enterics (23, 70, 139), there are some significant differences in the allosteric properties of the *Aeromonas* ADP-glucose pyrophosphorylase when compared to the enteric enzymes. In this case they indicated that the comparison of the allosteric properties of the ADP-glucose pyrophosphorylases in the various genera is more consistent with the 16S ribosomal RNA sequence studies of Fox et al (44).

The apparent similarities between the regulatory properties of the *Aeromonas* enzyme and those from *Mycobacteria,* an unrelated genus, with respect to activator specificity may possibly be due to convergent evolution. The *Mycobacteria* are aerobic organisms, whereas the aeromonads, although considered to be facultative anaerobes, give a strong positive test for cytochrome oxidase

activity, indicating a high capacity for oxidative metabolism. The availability of both fructose 6-P and fructose-P_2 as activators for these organisms may be more congruent with the greater capacity for aerobic metabolism in these organisms.

The similarities of the allosteric properties of the ADP-glucose pyrophosphorylases present in the genus *Rhodospirillum* suggest that a study of the allosteric properties of the enzyme can aid in the defining of taxonomic relationships.

It is also obvious from their properties that the green algal and higher plant ADP-glucose pyrophosphorylases (118) are very similar to the ADP-glucose pyrophosphorylase found in the blue-green bacteria (86). The clear evolutionary relationship of the blue-green bacteria to the green algae and higher plants has been pointed out previously with respect to their ability to evolve oxygen during photosynthesis and with respect to having similar photosynthetic pigments (155).

It would be of interest to compare the ADP-glucose synthetases from organisms of different genera at the immunological level to establish in further detail the relationships of the enzyme in the various genera as well as in the various activator classes. Some preliminary work has been done on this, using antibodies prepared against the purified ADP-glucose pyrophosphorylases of *Rhodopseudomonas sphaeroides* (175), *Rhodospirillum tenue* (174), and *E. coli* B (56; W. K. Kappel & J. Preiss, unpublished results) using enzyme activity neutralization techniques. Table 2 lists the data obtained with the antibody prepared against the *E. coli* enzyme. About 2.4 μl of antiserum per unit of enzyme inhibited the *E. coli* enzyme 50%. Maximal inhibition occurred at 26 μl per unit. The same sensitivity to neutralization was also seen towards the *E. coli* K12 enzyme. Moreover, the ADP-glucose pyrophosphorylases from the enteric group, having the same activator specificity, are almost as sensitive as the *E. coli* enzymes in their response to the antibody. All are inhibited almost 100%, and their relative titers range from 1.1 to 3.0. The most distantly related were the *C. freundii* and *E. tarda* enzymes. In contrast, the ADP-glucose pyrophosphorylases from organisms of other activator classes were either poorly or not inhibited at all by the *E. coli* enzyme antibody. The *Serratia* species, whose ADP-glucose pyrophosphorylase have no activator, are also not sensitive to the *E. coli* antibody. In contrast, the *E. hafniae* enzyme shows sensitivity that is similar to that seen with the other enteric ADP-glucose pyrophosphorylases activated by fructose 1,6-P_2, pyridoxal-P, or NADPH. This would suggest that the antigenic determinants of the antisera may be directed more to other domains of the protein rather than the activator binding domain.

Ouchterlony double immunodiffusion (105) studies were done (W. K. Kappel & J. Preiss, unpublished experiments) with some of the enteric organisms.

Table 2 Neutralization of various ADP-glucose pyrophosphorylases with *E. coli* anti–ADP-glucose pyrophosphorylase serum

Source of Enzyme	Activator(s)	Relative titer	% Maximum inhibition
E. aerogenes		1.5	98
E. aurescens		1.3	99
C. freundii		3.0	94
E. cloacae		1.7	94
S. dysenteriae	Fructose 1,6-P_2	1.1	98
E. tarda	Pyridoxal-P	2.9	94
S. typhimurium LT2	NADPH	1.1	98
S. enteriditis		2.2	92
K. pneumoniae		1.4	98
E. coli B		1.0	99
E. coli K12		1.0	99
R. tenue	Pyruvate	>30	0
A. viscosus	Pyruvate Fructose 6-P	>30	16
R. sphaeroides	Pyruvate Fructose 6-P Fructose 1,6-P_2	>30	0
A. liquefaciens	Fructose 6-P	6.9	82
A. hydrophila	Fructose 1,6-P_2	7.3	54
S. marcescens		>30	5
S. liquefaciens	None	>30	14
E. hafniae		2.0	98

Only one precipitin band with no spurs was found for the enzymes for *E. aurescens*, *S. dysenteriae*, and *E. coli* K12. Although precipitin bands were also noted for the *E. aerogenes*, *E. cloacae*, *S. typhimurium* LT-2, *S. enteriditis*, and *A. hydrophila* enzymes, spurs were also noted, suggesting some antigenic dissimilarities between these enzymes and the *E. coli* enzyme. The *S. liquefaciens*, *A. viscosus*, and spinach leaf enzymes displayed no precipitin band with the *E. coli* B antisera.

Similar results were obtained using the antisera prepared against *R. sphaeroides* (175) and *R. tenue* (174). Most ADP-glucose pyrophosphorylases were at least partially inhibited by the *R. tenue* antisera. However, with the exception of the *R. tenue* enzyme, the ADP-glucose pyrophosphorylases from either Rhodopseudomonads or other *Rhodospirillum* species are not inhibited more than 60% (174). The data indicated that the enzymes from the genus *Rhodospirillum* were immunologically more distinct than those from the enteric organisms.

In contrast, experiments with the antisera prepared against *R. sphaeroides* show that the enzyme obtained from other *Rhodopseudomonas* species was

effectively inhibited by the *R. sphaeroides* anti–ADP-glucose pyrophosphorylase with relative titers between 1 and 13.2 and maximum inactivation greater than 90%. The *E. coli* enzyme was inhibited only partially, and enzymes from the genus *Rhodospirillum* either were partially inhibited or not inhibited at all (175).

In summary, no clear association is seen between the strength of immunological cross-reactivity and the enzyme from the different activator classes. This may be explained by the fact that in the enzyme-neutralization studies, ADP-glucose–pyrophosphorylase activity became inactivated only when the binding of the antibody affected the enzyme's catalytic site. Therefore, when the values of relative titer are close to one another, the correlation of these values with the degree of primary amino acid sequence homology between proteins may be difficult to ascertain. To compare the degree of cross-reactivity of all of the antigenic determinants of the proteins, it may be useful to use the microcomplement fixation method (112), in addition to the enzyme neutralization method used in this study. Combined results from these studies should be more accurate in correlating immunological cross-reactivity and the amino acid sequence homology of the ADP-glucose synthetases from different activator classes than the results obtained from the enzyme-neutralization studies alone. The full understanding of the evolutionary relationships of the various allosteric activator classes would certainly be clarified by comparing the nucleotide sequences of the structural genes of the different ADP-glucose pyrophosphorylases.

BIOCHEMICAL STUDIES OF THE ACTIVATOR BINDING SITE OF *E. COLI* ADP-GLUCOSE PYROPHOSPHORYLASE

Specificity of Activation

Many studies have shown that the structural requirements for the allosteric activator site of the *E. coli* ADP-glucose pyrophosphorylase can be satisfied by compounds having two phosphate residues spatially situated apart as observed in fructose 1,6-P_2. Thus, sedoheptulose, 1,7-bisphosphate, 1,6-hexanediol-P_2 and glycerol 1,3-P_2 have been shown to be effective activators of the enzyme (113–116, 119). NADPH may also be viewed as being an analog of fructose 1,6-P_2 because a portion of its structure can be considered as ribose 2,5-P_2. Other activators, however, contain only one phosphate plus an aldehyde or a carboxyl group (pyridoxal-P, erythrose 4-P, 4 pyridoxic acid-5-P, 2-P-glycerate and phosphoenolpyruvate).

Most probably, basic amino acid residues in the activator binding site are involved in the binding of the activators, and thus binding of the potential activators requires that they contain at least one phosphate residue and another

anionic group, carboxyl or phosphate. If one of the residues is an epsilon amino group of lysine, an aldehyde group may satisfy one of the binding requirements by Schiff base formation. Three-dimensional space-filling models indicate that the distance between the two phosphate groups of fructose 1,6-P_2 either in furanose or open chain form is approximately equal to the distance from the aldehyde group to the phosphate group in pyridoxal-P.

Evidence for all these diverse metabolites binding to the same site has been obtained via kinetic (47, 129) and binding studies (54) and has been reviewed (130).

Evidence for the basic amino acid residues being involved has been obtained by chemical modification of the *E. coli* enzyme with phenylglyoxal (arginine residues) (12) and with trinitrobenzenesulfonate (lysine residues) (11).

Chemical Modification of the Activator Site of the E. coli *ADP-glucose Pyrophosphorylase*

The *E. coli* ADP-glucose pyrophosphorylase is covalently modified with pyridoxal-P by reduction with $NaBH_4$ (57, 107, 108), and an ϵ-amino group of a lysine residue in the protein was involved in the binding of the pyridoxal-P (57). The modified enzyme is significantly active and the enzyme no longer requires the presence of allosteric activator in reaction mixtures for high activity after incorporation of 0.5 mol of pyridoxal-P per mol of subunit (107). This suggests that the pyridoxal-P ligand is bound at the activator site. The presence of allosteric activators, fructose 1,6-P_2, and 1,6-hexanediol-P_2, as well as the inhibitor, 5'-AMP, protected against incorporation of pyridoxal-P, which also suggests that the pyridoxal-P was bound at the activator site. AMP, the inhibitor, had been shown subsequently in binding experiments (54) to prevent binding of activators to the enzyme. The prevention of binding of AMP is not due, however, to its binding to the same site as the activators, because AMP does inhibit the chemically modified enzyme in the same way as the unmodified enzyme in the presence of activator with the same $I_{0.5}$ value (concentration required to give 50% inhibition) (107). Thus the activator and inhibitor must bind at distinct sites.

Because pyridoxal-P can be covalently bound to the *E. coli* ADP-glucose pyrophosphorylase by $NaBH_4$ reduction, and because it is binding mainly at the allosteric site, it was of interest to determine the amino acid sequence about the phosphopyridoxyllysine residue.

After incorporation of [^{3}H]pyridoxal-P, the enzyme was degraded with CNBr. The pyridoxyl-P–containing peptide, which had over 70% of the total pyridoxal-P incorporated, was isolated and purified. Amino acid sequence analysis of the peptide was done using an automated Beckman Model 890C sequenator (72, 108) and is seen in Figure 3.

It consists of 57 amino acid residues with a molecular weight of about 6600. The amino terminal of the peptide had a 17-residue sequence overlap with the


```
                    5                   10                  15
NH2-Leu-Ala-Arg-Gln-Leu-Pro-Leu-Lys-Ser-Val-Ala-Leu-Ile-Leu-Ala-
                   20                  25                  30
Gly-Gly-Arg-Gly-Thr-Arg-Leu-Lys-Asp-Leu-Thr-Asn-Lys*-Arg-Ala-
                   35                  40                  45
Lys-Pro-Ala-Val-His-Phe-Gly-Gly-Lys-Phe-Arg-Ile-Ile-Asx-Phe-
                  50
Ala-Leu-Ser-Asn-CmCys-Ile-Asn-Ser-Gly- X - Ile-(Arg)
```

Figure 3 Primary sequence of the peptide containing the allosteric site of the *E. coli* ADP-glucose pyrophosphorylase after CNBr degradation. The lysine that has pyridoxal-P bound to it is marked with an asterisk. The CNBr peptide analyzed starts at residue 11 from the amino-terminal end and has a 17-residue sequence overlap with a previously determined amino-terminal sequence of the native enzyme (56).

previously determined amino-terminal sequence of the native enzyme (56). Thus, the lysine residue binding the pyridoxal-P was identified as residue 38 from the native enzyme's amino terminus.

There is a predominance of lysine and arginine residues in the sequence especially in close proximity to the phosphopyridoxylated lysine. The activator site has six positively charged amino acid residues about the phosphopyridoxylated lysine: arginine at residues 28, 31, and 39; lysine at residues 33, 38, and 41. As indicated before, two basic residues of the protein have been suggested as required for activator binding, and thus the sequence from residues 28 through 41 has the potential for containing residues to bind either the phosphates, carboxyl, or the aldehyde groups of the activator.

The various arginine residues at the allosteric site should be noted. Arginine residues have been shown to serve as positively charged recognition sites for negatively charged substrates and anionic cofactors in several enzymes (87, 136); one characteristic of arginine residues in protein is postulated to be its ability to bind phosphorylated metabolites (24). As indicated before, chemical modification with phenylglyoxal suggested the presence of an essential arginine at the allosteric activator site (11). The modification resulted in a lessened ability of the activator fructose 1,6-P_2 to stimulate activity. The apparent affinity for fructose 1,6-P_2 and the V_{max} of the reaction at saturating concentrations of fructose 1,6-P_2 were decreased by the arginine modification. Both 1,6 hexanediol-P_2 and fructose 1,6-P_2 were able to partially protect the enzyme from inactivation. Additional studies are required to determine if the modified arginine is indeed one of the residues 28, 31, or 39 or is elsewhere in the primary sequence of the subunit.

Comparison of the Amino-Terminal Sequences of Other Bacterial ADP-glucose Pyrophosphorylases with the E. coli *ADP-glucose Pyrophosphorylase*

Because the allosteric activator site was found near the amino-terminal of the *E. coli* subunit, it was of interest to see if any similarities between the amino-terminal sequences of bacterial ADP-glucose pyrophosphorylases in the same or in other activator classes could be found. Partial amino-terminal sequences of four bacterial enzymes, recently purified, with the *E. coli* enzyme sequence have been determined and are shown in Figure 4.

The ADP-glucose pyrophosphorylases from *E. coli* and *S. typhimurium*, two related organisms that have the same activator specificity, differ in the first 25 amino acids only at residues 8, 9, and 16. The differences at positions 8 and 9 could also be considered to be of conservative nature (histidine instead of arginine and leucine instead of valine) both in ionic character and hydrophobicity. Interchange of the amino acids only requires one base change in the nucleotide codon. The great conservation in amino acid sequence for the enzyme from these two organisms may be due to their close relatedness as well as the enzyme having the same allosteric activator specificity. Further comparison of the sequences would be required to determine if the allosteric binding sites are indeed similar. The immunological tests with antibodies produced against either enzyme show partial cross-reaction between the antibodies prepared against one enzyme with the other enzyme, indicating that there are some differences between the amino acid sequences of the two enzymes (Table 2) (56, 85).

The amino-terminal amino acid sequences of the enzymes of the photosynthetic organisms, *R. tenue*, *R. rubrum*, and *R. sphaeroides*, are quite different from those isolated from the enteric organisms as well as different from each other. The *R. sphaeroides* enzyme is also activated by fructose 1,6-P_2 in addition to fructose 6-P and pyruvate (122). Although it is activated by fructose 1,6-P_2, as are the enteric enzymes, the enzyme sequence has no resemblance to the enteric-enzyme amino acid sequences. Of interest is that the amino-terminal amino acid sequence of the two enzymes activated specifically by pyruvate, *R. tenue* and *R. rubrum*, are also quite dissimilar. Indeed, antibody prepared against the *R. tenue* enzyme had little effect on *R. rubrum* activity in enzyme neutralization tests. Only 25% of the enzyme activity was inhibited, suggesting very little similarity of the two enzymes in structure (174). Although all the ADP-glucose pyrophosphorylases from the genus *Rhodospirillum* have been found to be activated by pyruvate, the immunological studies show that their amino acid sequences may be distinct. Whether the allosteric activator site of the photosynthetic bacterial ADP-glucose pyrophosphorylase is situated near the amino-terminal portion of the photosynthetic bacterial enzyme protein, as seen for the enteric enzymes, is not known. Further studies are required to

Activator Specificity	Bacteria	NH_2 terminal sequence
		1 5 10 15 20
Fructose 1,6-P_2, NADPH, Pyridoxal-P	E. coli	Val-Ser-Leu-Glu-Lys-Asn-Asp-His-Leu-Met-Leu-Ala-Arg-Gln-Leu-Pro-Leu-Lys-Ser-Val-
Fructose 1,6-P_2, NADPH, Pyridoxal-P	S. typhimurium	Val-Ser-Leu-Glu-Lys-Asn-Asp-Arg-Val-Met-Leu-Ala-Arg-Glx-Leu-Glu-Leu-Lys-Ser-Val-
Fructose 1,6-P_2, Fructose 6-P, Pyruvate	R. sphaeroides	Met-Lys-Ala-Gln-Pro- X -Leu-Arg-Leu-Thr-Ala-Gln-Ala-Met-Ala-Phe-Val- . . . .
Pyruvate	R. rubrum	Leu-Asp-Gln-Ile-Thr-Glu-Phe-Gln-Leu-Asp-Ile-Asn-(Arg)-Ala-Leu-Thr-Glu- X - X -Ala-
Pyruvate	R. tenue	Pro-Val-Glu-Thr-Glu-Asn-Arg-Ser-Asp-Ser-Asp-Phe-Asp-Gln-Arg-Glu-His-Gly-Asp-Ser-
		21 25
	E. coli	Ala-Leu-Ile-Leu-Ala-Gly-Gly-Arg-Gly-Thr- . . . .
	S. typhimurium	Ala-Leu-Ile-Leu-Ala-(Gly)-Gly- X -(Gly)- . . . .
	R. sphaeroides	
	R. rubrum	
	R. tenue	Arg-Phe-Ile-Ser-Glx-Leu- X -Arg-(Asn)- . . . .

Figure 4 Amino-terminal sequences of the ADP-glucose pyrophosphorylases of *E. coli* (57), *Rhodopseudomonas sphaeroides* (175), *Rhodospirillum rubrum* (124), *Rhodospirillum tenue* (174), and *S. typhimurium* (85).

determine where the allosteric activator sites are situated in other bacterial ADP-glucose pyrophosphorylases. Recent experiments have shown that an analogue of pyruvate, bromopyruvate, can covalently modify the *R. sphaeroides* ADP-glucose pyrophosphorylase, and it appears that it is covalently linked to a cysteine residue in the protein at or near the activator site (130).

GENETIC REGULATION OF GLYCOGEN SYNTHESIS IN *E. COLI* AND IN *S. TYPHIMURIUM*

In the previous discussion concerning PP_i inhibition of ADP-glucose pyrophosphorylase, it was pointed out that the rate of glycogen synthesis in *E. coli* strains may increase 2- to 5-fold upon transition of the organisms from exponential phase of growth to stationary phase. This also occurs with *S. typhimurium* LT-2 (158) and with *Agrobacterium tumefaciens* (121) when grown in enriched media.

In enriched media containing 1% glucose, an 11- to 12-fold increase is observed in the specific activities of ADP-glucose pyrophosphorylase and glycogen synthase and a 5-fold increase in the levels of branching-enzyme activity. In minimal media containing 0.6% glucose with limiting NH_4^+ salt, the level of the above enzymes are less repressed in the exponential phase of growth. There is, however, still a 2- to 3-fold increase in the levels of ADP-glucose pyrophosphorylase and glycogen synthase when the cells reach stationary phase. Branching-enzyme activity is fully derepressed in the exponential phase in minimal media, and no further increase in stationary phase is observed. Although the derepression is not great in minimal media, the maximum levels of the biosynthetic enzymes in stationary phase are the same as in stationary phase after growth in enriched media. The derepression of glycogen synthase and ADP-glucose pyrophosphorylase is coordinate. The studies of Cattaneo et al (18) suggest that the increase is due to protein synthesis, because glycogen accumulation in *E. coli* at the beginning of stationary phase can be inhibited by addition of chloramphenicol to the medium.

As would be expected, derepression of the activity of the glycogen biosynthetic enzymes also occurs when the *S. typhimurium* LT-2 (158) and *Agrobacterium tumefaciens* (121) go from logarithmic to stationary phase in enriched media. The derepression of the three glycogen biosynthetic enzymes in *S. typhimurium* LT-2 is coordinate (158).

Mutant strains SG3 and AC70R1, isolated from *E. coli* B, are derepressed in the levels of glycogen synthase and ADP-glucose pyrophosphorylase during exponential growth (50, 103, 113, 119, 121). In enriched media, mutant SG3 can accumulate twice as much glycogen per gram of wet weight cells in stationary phase and contains 8- to 11-fold more ADP-glucose–pyrophosphorylase activity and 3-to 4-fold as much glycogen synthase activity

as the parent strain in the exponential phase of growth. However, branching-enzyme activity is the same. In mutant AC70R1, there is almost 10-fold as much glycogen accumulated in the stationary phase, along with 11, 5.5, and 2.6 times as much ADP-glucose pyrophosphorylase, glycogen synthase, and branching enzyme, respectively, in the exponential growth phase in enriched media. A further elevation occurs when both mutants enter stationary phase. With SG3, a 2- to 3-fold increase in the specific activities of ADP-glucose pyrophosphorylase and glycogen synthase, and in mutant AC70R1, a 5- to 10-fold increase in the specific activities of the 3 enzymes are observed. In minimal media essentially similar results are observed with mutants SG3 and AC70R1.

Glycogen accumulation rates are greater in AC70R1 than in either SG3 or *E. coli* B, and this is consistent with the finding of higher levels of the glycogen biosynthetic enzymes seen in mutant AC70R1 as compared to mutant SG3 and the parent wild-type strain, *E. coli* B. The enzymes, ADP-glucose pyrophosphorylase and glycogen synthase, in the derepressed mutants are identical to the parent *E. coli* B enzymes in their kinetic properties (45) and in the case of the ADP-glucose pyrophosphorylase in their regulatory properties (55, 56).

The reactions of the *E. coli* B enzymes against antisera prepared against the analogous glycogen biosynthetic enzymes from AC70R1 in neutralization of activity tests as well as in double immunodiffusion gels are identical to those observed for the AC70R1 enzymes (56, 68). Therefore, more normal enzyme seems to be synthesized than enzyme molecules that have greater catalytic activity.

Possible Regulation by Cyclic AMP

Very little is known about the mechanism of genetic regulation. There are, however, some preliminary experiments that suggest that cyclic AMP (cAMP) and guanosine 3'5'-bis(diphosphate) (ppGpp) may be involved in regulation of glycogen accumulation in *E. coli*. The possibility of ppGpp involvement was discussed previously in this review.

Dietzler et al (39, 40) have found that addition of cAMP to cultures of *E. coli* W4597(K) that utilize glucose as a carbon source stimulated the rate of glycogen accumulation during the exponential phase of growth up to 5-fold, and in stationary phase about 1.5-fold. Cyclic AMP had no effect on the levels of the metabolites, ATP, AMP, fructose 1,6-P_2, and glucose 6-P or on energy charge. Cyclic AMP is not an allosteric effector of ADP-glucose pyrophosphorylase (49, 129). Thus, Dietzler et al (39, 40) have postulated an indirect role for cAMP in regulating glycogen synthesis by changing the cellular level of a presently unknown inhibitor of the ADP-glucose pyrophosphorylase.

The structural genes for the glycogen biosynthetic genes have recently been cloned (103), enabling the study of possible regulatory phenomena associated

with the transcriptional and translational events of these enzymes in vitro. Preliminary studies have shown the in vitro expression of the ADP-glucose–pyrophosphorylase structural gene of *E. coli*, *glg*C, using the procedure of measuring the formation of the first dipeptide of the gene product (137). They also suggest that cAMP and cAMP receptor protein are involved in the expression of the *glg*C gene (166). Cyclic AMP and cAMP receptor protein, when added together, stimulated the synthesis of fMet-Val and fMet-Val-Ser about 2.5- to 4-fold using the plasmid pOP12 containing the *glg*C gene. The dipeptide and tripeptide formed are the amino-terminal dipeptide and tripeptides of the *glg*C gene. Other metabolites thought possibly to affect glycogen synthesis at the gene level were tested in vitro and found to be inactive. Fructose 1,6-P_2 (0.5 mM), glucose 6-P (0.6 mM), and ppGpp (0.15 mM) had no detectable effect upon fMet-Val synthesis when added either separately or in various combinations (166).

The observed stimulation is significant but still not very large, being only about 3-fold. However, the isolation of the aforementioned mutants derepressed in the levels of glycogen synthase and ADP-glucose pyrophosphorylase suggest the possible presence of a repressor protein involved in regulation of the expression of the structural genes of glycogen synthase and ADP-glucose pyrophosphorylase. It is quite possible the cAMP and cAMP receptor protein are involved in the modulation of *glg*A and *glg*C expression by modulation of the repression.

Recent results by Leckie et al (82) are consistent with above results. They used *cya* and *crp* mutants of *E. coli*, deficient in adenylate cyclase and in cAMP receptor protein, and showed that in both cases the rates of glycogen synthesis were lower than that of the parent strains. Addition of cAMP to the media of the *crp* mutants did not give stimulation of glycogen synthetic rates, and thus the stimulation by cAMP originally observed in the parent strains was dependent on the presence of the cAMP receptor protein.

It appears the synthesis of the glycogen biosynthetic enzymes is apparently under complex regulation that may involve regulation of synthesis of cAMP and ppGpp. This is understandable, because it is influenced not only by nutrient deprivation in the presence of excess carbon but also by the rate of bacterial growth (75, 119, 121, 127).

Location of the Glycogen Genes in the Linkage Maps of E. coli *B*, E. coli *K12, and* S. typhimurium

Cattaneo and her colleagues have isolated glycogen synthase, branching enzyme and ADP-glucose pyrophosphorylase mutants of *E. coli* K12 (17, 29, 31) and showed that the mutations were all cotransducible with the maltose A genes (151). Subsequently the same group reported that the *glg* genes are cotransducible with the aspartate semialdehyde gene *asd* (17). The relative order of these

glg genes in *E. coli* K12 were established via transduction tests that suggested the following order at 75 min on the linkage map: *mal*A-*glg*A (glycogen synthase)-*glg*C (ADP-glucose pyrophosphorylase)-*glg*B (branching enzyme)-*asd* (81).

The catabolite L-glycerol 3-phosphate dehydrogenase gene, *glp*D, is located very close to the maltose A and *asd* genes on the *E. coli* genetic linkage map (25). Thus a *glp*D mutant of *E. coli* was isolated and used to show a cotransduction of about 80% with the structural genes of the glycogen biosynthetic enzymes, *glg*A, B, and C (127). The SG3 mutation, which gives rise to derepressed levels of ADP-glucose pyrophosphorylase and glycogen synthase, also cotransduced about 80% with *glp*D. However, the AC70R1 mutation, which elicits derepressed levels of the three glycogen biosynthetic enzymes, appeared not to be linked with the *glg*A, B, C genes, or *glp*D (13).

In *Salmonella typhimurium* LT-2 the cotransduction frequencies of *glg*A and *glg*C with *asd* and *glp*D suggested the gene order *glp*D-*glg*A-*glg*C-*asd* (158). The results of three factor crosses were consistent with this proposed order. Thus the gene order for at least *glg*A and *glg*C is the same as seen in *E. coli* K12.

In addition, two mutants of *S. typhimurium* LT-2, JP54 and JP74, which accumulated glycogen 1.5- to 2-fold as fast as the wild-type organisms, contain 1.5–2-fold the levels of the three glycogen biosynthetic enzymes (K. Steiner & J. Preiss; unpublished results). These mutations, defined as *glg*R, were also linked to *glp*D and *asd*. Another mutant, JP102, was derepressed about 2- to 4-fold as high in the levels of the three glycogen enzymes, pyrophosphorylase, synthase, and branching enzyme. However, this mutation was unlinked with the *glg* genes in cotransduction studies to either *asd* or *glp*D.

The *glg* structural genes may exist in an operon. Various observations support this concept: (*a*) The genes are clustered on the *E. coli* and *S. typhimurium* chromosomes. (*b*) The activities of two of the enzymes, ADP-glucose pyrophosphorylase and glycogen synthase, are coordinately derepressed at the end of the exponential growth phase in *E. coli* B (75) and *E. coli* K12 (103, 104). (*c*) The three glycogen biosynthetic enzymes are coordinately derepressed in *S. typhimurium* LT-2 at the end of the exponential growth phase (158). Glycogen-excess mutants in *E. coli* B and in *S. typhimurium* LT-2 appear to be derepressed for the synthesis of both glycogen synthase and ADP-glucose pyrophosphorylase and/or branching enzyme. This would suggest that these mutants have received a mutation in one of the control sites for the operon. The signal for the increase of the glycogen synthetic enzymes in *E. coli* appears to be linked with a decrease in the growth rate. As cell division ceases, the enzymes are synthesized to store any available excess carbon as a reserve. It would be of interest to determine the mechanism of the genetic regulation of the biosynthesis of these enzymes, as it may give us some insight into the processes

by which the microorganism is able to modulate various processes in conjunction with the modulation of its growth rate and various processes occurring in stationary phase.

Although the genetic regulation of the biosynthesis of the glycogen biosynthetic enzymes is an important factor in controlling the accumulation of glycogen in *E. coli* and *S. typhimurium* LT-2, it should be pointed out that in glucose-minimal media there is already an 8- to 20-fold excess of the ADP-glucose pyrophosphorylase and glycogen synthase levels in the exponential phase of growth to account for the maximum rate of glycogen accumulation seen in the stationary phase. The activities of these enzymes must therefore be controlled by the intracellular concentrations of their substrates and effectors. This is consistent with the evidence indicating that the allosteric regulation of ADP-glucose pyrophosphorylase is physiologically significant in the control of the rate of glycogen synthesis in *E. coli* and in *S. typhimurium*.

Cloning of the Glycogen Biosynthetic Enzyme Structural Genes of E. coli

Recombinant DNA techniques were initiated to clone a region of the *E. coli* chromosome containing the structural genes of glycogen biosynthetic enzymes to aid in our understanding of the regulation of *glg* gene expression in bacteria at the molecular level.

The structural genes were isolated via "shotgun" cloning *E. coli* DNA, i.e. large DNA fragments obtained by partial restiction endonuclease digestion were cloned onto the bacterial plasmid pBR322, and those clones containing the *glg* genes were isolated by cotransformation with a neighboring selectable gene, *asd* (103, 104).

The *E. coli* K12 strain used in the initial cloning experiment was mutant G6MD3 that has deletion from the *mal*A region through *asd* and hence lacks the putative *glg* operon (29, 144).

It was found that partial digestion of *E. coli* K12 chromosomal DNA with the restriction nuclease, *Pst*I, and insertion of the DNA fragments at the *Pst*I site of pBR322 yielded plasmids containing the *glg* genes, A, B, and C, as well as *asd*. The whole DNA fragment containing the 4 genes was about 10.5 kilobases long; a schematic representation is shown in Figure 5.

One recombinant plasmid, designated pOP12, was physically mapped with 9 restriction endonucleases (2, 103, 130). On the basis of subcloning *Bam*HI and *Pst*I fragments and by deletion mapping of *Eco*RI or *Pst*I, partial digestion of the 10.5-kilobase pair fragment, and subcloning these fragments the order of the 4 genes was determined (2, 103, 130); it confirmed the order obtained by transduction experiments as *asd-glg*B-*glg*C-*glg*A (81).

Strains bearing the pOP12 plasmid were found to overproduce the product of *asd* gene, aspartate semialdehyde dehydrogenase, 6- to 65-fold, depending on the growth conditions used (126).

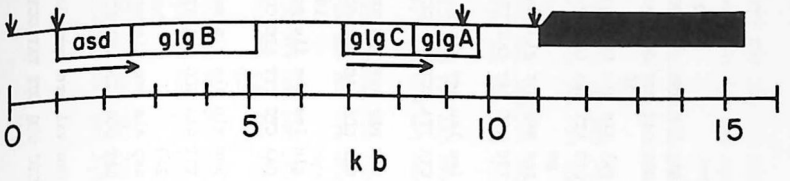


Figure 5 Schematic presentation of the 10.5 kb DNA fragment containing the *glg*A, B, C, and *asd* genes of *E. coli* K12. The genes were mapped by digestion with nine restriction endonucleases having one or two sites in pBR322 and by deletion mapping by partial digestion with *Eco*RI or *Pst*I and subcloning the resultant fragments as previously described (2, 103). The exact location of the *glg*B gene is based on the reported nucleotide sequence of the *asd* gene (59) and on the N-terminal amino acid sequence of the branching enzyme (68). The *vertical arrows* indicate the cleavage sites of *Pst*I while the *horizontal arrows* indicate the direction of transcription.

Transformation of *E. coli* K12 with pOP12 resulted in a 30-fold increase in the specific activity of ADP-glucose pyrophosphorylase, the *glg*C product, but increased the *glg*A and *glg*B products only 8- and 10-fold, respectively (103). Transformation of the *E. coli* B derepressed mutant, AC70R1, elevated the specific activity of the 3 glycogen biosynthetic enzymes to about 30- to 40-fold (103). One can observe the presence of these enzymes in crude extracts of the transformed cells on gels after SDS electrophoresis, after staining with Coomassie blue, and these enzymes may constitute about 10–15% of the total cellular proteins. The enzymes are not observed in the SDS gels of crude extracts of the normal AC70R1 cells. The increase in levels of the glycogen biosynthetic enzyme activity are thus due to gene dosage effects and not due to activation of preexisting proteins.

Moreover, various experiments have shown that ADP-glucose pyrophosphorylase and glycogen synthase purified from AC70R1 transformed with pOP12 is found to be similar, if not identical, with wild-type enzymes with respect to enzymatic, immunological, and physical properties (103; E. Greenberg & J. Preiss; unpublished results).

Because the NH_2-terminal amino acid sequences were known for the glycogen biosynthetic enzymes as well as for aspartate semialdehyde dehydrogenase, the precise positions of the translational starts of their structural genes on the DNA fragment were determined from the results of Haziza et al (59) and from our recent results.

Richaud et al (135) were able to identify the *asd* gene in an *Eco*RI library of the *E. coli* genome, cloned in λgtλB, and transfer the 4.5-kilobase fragment into the *Eco*RI site of pBR322. Haziza et al (58) were then able to further reduce the size of the fragment to 1.65 kilobases and subclone it to yield a plasmid still containing the complete *asd* gene. The nucleotide sequence was determined, and the sequence predicted a polypeptide chain of 367 amino acids. The translational start was at base pair 240 of their fragment, and the deduced amino acid sequence was consistent with the sequence of 35 amino acids of the NH_2-terminal and the finding of alanine at the carboxyl terminal end that

Fig. 6

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Fig. 6 (continued)

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                                                570
                                                                580
                                                                                590
leu leu phe met gly asn glu phe ala gln gly arg glu trp asn his asp ala ser leu asp trp his leu leu glu gly gly asp asn trp his
CTA CTG TTC ATG GGT AAC GAA TTT GCC CAG GGC CGC GAG TGG AAC CAT GAC GCC AGC CTC GAC TGG CAT CTG TTG GAA GGC GGC GAT AAC TGG CAC
                                                600
                                                                610
                                                                                620
his gly val gln arg leu val arg asp leu asn leu thr tyr arg his his lys ala met his glu leu asp pro asp pro tyr gly phe glu trp
CAC GGT GTC CAG CGT CTG GTG CGC GAT CTG AAC CTC ACC TAC CGC CAC CAT AAA GCA ATG CAT GAA CTG GAC CCC GAC CCG TAC GGC TTT GAA TGG
                                                630
                                                                640
                                                                                650
leu val val asp asp lys glu arg ser val leu ile phe val arg arg asp lys glu gly asn glu ile val ala ser asn phe thr pro val
CTG GTG GTG GAT GAC AAA GAA CGC TCG GTG CTG ATC TTT GTG CGT CGC GAT AAA GAG GGT AAC GAA ATC ATC GTT GCC AGT AAC TTT ACG CCG GTA
                                                                670
                                                                                680
                                                                                                690
pro arg his asp tyr arg phe gly ile asn gln pro gly lys trp arg glu ile leu asn thr asp ser met his tyr his gly ser asn ala gly
CCG CGT CAT GAT TAT CGC TTC GGC ATA AAC CAG CCG GGC AAA TGG CGT GAA ATC CTC AAT ACC GAT TCC ATG CAC TAT CAC GGC AGT AAT GCT GGC
                                                700
                                                                710
                                                                                720
asn gly gly thr val his ser asp glu ile ala ser his gly arg gln his ser leu ser leu thr leu pro pro leu ala thr ile trp leu val
AAT GGC GGC ACG GTA CAC AGC GAT GAG ATT GCC AGC CAC GGT CGT CAG CAT TCA CTA AGC CTG ACG CTA CCA CCG CTG GCC ACT ATC TGG CTG GTT

arg glu ala glu stop CACAACTCGCCATTGGCAA
CGG GAG GCA GAA TGA
```

Figure 6 The nucleotide and deduced amino acid sequence of *glg*B gene (P. A. Baecker & J. Preiss, unpublished experiments). The sequence shown is that of the antisense strand and starts at base 1580 of the *Bam*HI fragment reported by Haziza et al (59). The translational start of the *glg*B gene on their fragment is at nucleotide 1616. The amino acid sequence starting with serine is the reported amino-terminal sequence of the branching enzyme (68), and the underlined portion of the deduced amino acid sequence was previously determined by peptide sequencing methods (68) and is consistent with the nucleotide sequence found.

had been previously determined (126). Of further interest is that the translational start of the *glg*B is at nucleotide 1616 (Figure 6). This was deduced by us from our determination of the NH_2-terminal sequence of branching enzyme, the *glg*B product (68). The complete nucleotide sequence of the *glg*B gene is also seen in Figure 6.

The deduced amino acid sequence seen at the amino terminal end of the enzyme is consistent with the previously determined peptide sequence found for the amino terminal end of the enzyme.

The deduced amino acid sequence is also consistent with the amino acid analysis of the purified protein (68; E. Greenberg & J. Preiss; unpublished results). Thus the branching enzyme is composed of 727 amino acids, not including the initial methionine, with a molecular weight of 84,048. That molecular weight is in agreement with the reported molecular weight of 84,000 (6).

The nucleotide sequence of the *glg*C gene coding for ADP-glucose pyrophosphorylase using for the most part the methodology of Maxam & Gilbert (93) has also been elucidated and is shown in Figure 7. It consists of 1293 base pairs specifying a protein of 431 amino acids. This amino acid sequence deduced from the DNA sequence is also consistent with the known amino terminal amino acid sequence and the amino acid composition of the enzyme (56). The calculated molecular weight of the deduced protein is 48,762, which is in excellent agreement with the determined approximate molecular weight of 50,000 (55, 56).

Of interest are the relative positions of the lysine residues that were chemically modified by pyridoxal-P and previously discussed. From amino acid sequences the lysine involved in binding the allosteric activators was known to be the 38th amino acid from the amino-terminal valine. In Figure 7 it is shown as amino acid residue 39 from the amino-terminal methionine residue. Obviously the amino-terminal methionine residue is subsequently removed from the native protein because the protein isolated from *E. coli* lacks the N-terminal methionine (56, 72, 108). The lysine residue that is protected from reductive phosphopyridoxylation by ADP-glucose plus Mg^{++} (108) is now identified as residue 195.

Experiments are currently in progress to identify the amino acid residues in close proximity to the inhibitor binding site (80). The knowledge of the complete amino acid sequence would enable us to determine the relative position of the inhibitor from the activator and catalytic sites. This in combination with other studies may shed light on the secondary structure and conformation of the ADP-glucose pyrophosphorylase and provide good insight into the functional aspects of the structure of the enzyme.

Figure 7 also shows that the open reading frame for the *glg*C gene product terminates with the sequence TAATG. However, another open reading frame is found beyond the *glg*C gene that utilizes part of the termination sequence for

Fig. 7

```
1                                   10                                  20                                  30
met val ser leu glu lys asn asp his leu met leu ala arg gln leu pro leu lys ser val ala leu ile leu ala gly gly arg gly
ATG GTT AGT TTA GAG AAG AAC GAT CAC TTA ATG TTG GCG CGC CAG CTG CCA TTG AAA TCT GTT GCC CTG ATA CTG GCG GGA GGA CGT GGT

                                    40                                  50                                  60
thr arg leu lys asp leu thr asn lys*arg ala lys pro ala val his phe gly gly lys phe arg ile ile asp phe ala leu ser asn
ACC CGC CTG AAG GAT TTA ACC AAT AAG CGA GCA AAA CCG GCC GTA CAC TTC GGC GGT AAG TTC CGC ATT ATC GAC TTT GCG CTG TCT AAC

                                    70                                  80                                  90
cys-ile asn ser gly ile arg arg met gly val ile thr gln tyr gln ser his thr leu val gln his ile gln arg gly trp ser phe
TGC ATC AAC TCC GGG ATC CGT CGT ATG GGC GTG ATC ACC CAG TAC CAG TCC CAC ACT CTG GTG CAG CAC ATT CAG CGC GGC TGG TCA TTC

                                   100                                 110                                 120
phe asn glu glu met asn glu phe val asp leu leu pro ala gln gln arg met lys gly glu asn trp tyr arg gly thr ala asp ala
TTC AAT GAA GAA ATG AAC GAG TTT GTC GAT CTG CTG CCA GCA CAG CAG AGA ATG AAA GGG GAA AAC TGG TAT CGC GGC ACC GCA GAT GCG

                                   130                                 140                                 150
val thr gln asn leu asp ile ile arg arg tyr lys ala glu tyr val val ile leu ala gly asp his ile tyr lys gln asp tyr ser
GTC ACC CAA AAC CTC GAC ATT ATC CGT CGT TAT AAA GCG GAA TAC GTG GTG ATC CTG GCG GGC GAC CAT ATC TAC AAG CAA GAC TAC TCG

                                   160                                 170                                 180
arg met leu ile asp his val glu lys gly val arg cys thr val val cys met pro val pro ile glu glu ala ser ala phe gly val
CGT ATG CTT ATC GAT CAC GTC GAA AAA GGT GTA CGT TGT ACC GTT GTT TGT ATG CCA GTA CCG ATT GAA GAA GCC TCC GCA TTT GGC GTT

                                   190                                 200                                 210
met ala val asp glu asn asp lys thr ile glu phe val glu lys*pro ala asn pro pro ser met pro asn asp pro ser lys ser leu
ATG GCG GTT GAT GAG AAC GAT AAA ACT ATC GAA TTC GTG GAA AAA CCT GCT AAC CCG CCG TCA ATG CCG AAC GAT CCG AGC AAA TCT CTG

                                   220                                 230                                 240
ala ser met gly ile tyr val phe asp ala asp tyr leu tyr glu leu leu glu glu asp asp arg asp glu asn ser ser his asp phe
GCG AGT ATG GGT ATC TAC GTC TTT GAC GCC GAC TAT CTG TAT GAA CTG CTG GAA GAA GAC GAT CGC GAT GAG AAC TCC AGC CAC GAC TTT
```

Fig. 7 (continued)

```
                                   250                                 260                                 270
gly lys asp leu ile pro lys ile thr glu ala gly leu ala tyr ala his pro phe pro leu ser cys val gln ser asp pro asp ala
GGC AAA GAT TTG ATT CCC AAG ATC ACC GAA GCC GGT CTG GCC TAT GCG CAC CCG TTC CCG CTC TCT TGC GTA CAA TCC GAC CCG GAT GCC

                                   280                                 290                                 300
glu pro tyr trp arg asp val gly thr leu glu ala tyr trp lys ala asn leu asp leu ala ser val val pro lys leu asp met tyr
GAG CCG TAC TGG CGC GAT GTG GGT ACG CTG GAA GCT TAC TGG AAA GCG AAC CTC GAT CTG GCC TCT GTG GTG CCG AAA CTG GAT ATG TAC

                                   310                                 320                                 330
asp arg asn trp pro ile arg thr tyr asn glu ser leu pro pro ala lys phe val gln asp arg ser gly ser his gly met thr leu
GAT CGC AAT TGG CCA ATT CGC ACC TAC AAT GAA TCA TTA CCG CCA GCG AAA TTC GTG CAG GAT CGC TCC GGT AGC CAC GGG ATG ACC CTT

                                   340                                 350                                 360
asn ser leu val ser gly gly cys val ile ser gly ser val val val gln ser val leu phe ser arg val arg val asn ser phe cys
AAC TCA CTG GTT TCC GGC GGT TGT GTG ATC TCC GGT TCG GTG GTG GTG CAG TCC GTT CTG TTC TCG CGC GTT CGC GTG AAT TCA TTC TGC

                                   370                                 380                                 390
asn ile asp ser ala val leu leu pro glu val trp val gly arg ser cys arg leu arg arg cys val ile asp arg ala cys val ile
AAC ATT GAT TCC GCC GTA TTG TTA CCG GAA GTA TGG GTA GGT CGC TCG TGC CGT CTG CGC CGC TGC GTC ATC GAT CGT GCT TGT GTT ATT

                                   400                                 410                                 420
pro glu gly met val ile gly glu asn ala glu glu asp ala arg arg phe tyr arg ser glu glu gly ile val leu val thr arg glu
CCG GAA GGC ATG GTG ATT GGT GAA AAC GCA GAG GAA GAT GCA CGT CGT TTC TAT CGT TCA GAA GAA GGC ATC GTG CTG GTA ACG CGC GAA

                                   430
met leu arg lys leu gly his lys gln glu arg stop
ATG CTA CGG AAG TTA GGG CAT AAA CAG GAG CGA TAATG CAG GTT TTA CAT GTA TGT TCA GAG ATG TTT . . .
                                                    met gln val leu his val cys ser glu met phe . . . (glycogen synthase)
```

Figure 7 Nucleotide and deduced amino acid sequence of *glg*C as determined by Baecker & Preiss (2). The antisense strand sequence is shown. The amino acid sequences underlined are those that had been determined by amino acid sequencing previously (56, 72, 108). The asterisk at lysine residues 39 and 195 are those that had been shown to be phosphopyridoxylated with pyridoxal-P plus $NaBH_4$ (108). The amino acid sequence seen after the stop codon TAA after the carboxyl terminal residue, Arg, is the amino-terminal sequence of glycogen synthase (68).

initiation of translation of another polypeptide in the new reading frame. A potential Shine-Dalgarno sequence (150), AGGAG, is found at the end of the *glg*C gene and may serve as a ribosomal binding site for this second gene. The deduced NH_2-terminal sequence of this second gene product found after the TAA termination sequence of the *glg*C is also seen in Figure 7 and the deduced sequence of 11 residues is identical to the NH_2-terminal amino acid sequence reported for the *E. coli* glycogen synthase (68). Thus it would appear the ADP-glucose pyrophosphorylase and glycogen synthase are translated from a singly polycistronic message. Previous studies have shown that ADP-glucose pyrophosphorylase and glycogen synthase are coordinately regulated (75, 119, 121), and thus it is not altogether surprising that the message for both gene products is transcribed in one mRNA.

The extreme conservation of genetic material shown in the intercistronic region between the two genes, *glg*C and *glg*A, has been observed previously (173) for the nucleotide sequence of polycistronic *trp* mRNA as deduced from the DNA sequence.

Thus with the isolation of the structural genes for the glycogen biosynthetic enzymes, a more detailed study of the genetic regulation of the biosynthesis of these enzymes is approachable. It would be of interest to know if the 1.8-kilobase area seen in Figure 5 of the isolated DNA fragment between the *glg*B and *glg*C genes codes for other proteins that perhaps may also be pertinent for the synthesis of glycogen and perhaps for regulation of the glycogen biosynthetic enzymes. One possibility is that this area may be involved in the expression of a repressor protein for *glg*C and *glg*A. Obviously the nucleotide sequence preceding the translation starts would be of prime interest, because it should encompass the promoter-operator region. It would also be of interest if the nucleotide sequence of the promoter-operator region has some similarity to other promoters that bind cAMP and the cAMP receptor protein, and whether there are two promoter-operator regions; one for the *glg*B gene and the other for the *glg*A and C genes. The availability of this portion of DNA containing the structural genes of the glycogen biosynthetic enzyme presents to us a number of approaches that may enable us to determine the mechanism of genetic regulation of the glycogen operon.

Literature Cited

1. Atkinson, D. E. 1970. *The Enzymes* 1:461–89
2. Baecker, P. A., Preiss, J. 1983. *J. Biol. Chem.* 258:5084–88
3. Bascomb, S., Lapage, S. P., Willcox, W. R. 1971. *J. Gen. Microbiol.* 66:279–95
4. Bochner, B. R., Ames, B. N. 1982. *J. Biol. Chem.* 257:9759–69
5. Bornefeldt, T. 1981. *Arch. Microbiol.* 129:371–73
6. Boyer, C., Preiss, J. 1977. *Biochemistry* 16:3693–99
7. Brana, A. F., Manzanal, M. B., Hardisson, C. 1980. *J. Bacteriol.* 144:1139–42
8. Brana, A. F., Manzanal, M. B., Hardisson, C. 1982. *Can. J. Microbiol.* 28:1320–23
9. Bridger, W. A., Paranchych, W. 1978. *Can. J. Biochem.* 56:403–6
10. Burleigh, I. G., Dawes, E. A. 1967. *Biochem. J.* 102:236–50

11. Carlson, C. A., Preiss, J. 1981. *Biochemistry* 20:7519–28
12. Carlson, C. A., Preiss, J. 1982. *Biochemistry* 21:1929–34
13. Carlson, C. A., Parsons, T. F., Preiss, J. 1976. *J. Biol. Chem.* 251:7866–92
14. Carnal, W. N., Black, C. C. 1979. *Biochem. Biophys. Res. Commun.* 86: 20–26
15. Cashel, M. 1975. *Ann. Rev. Microbiol.* 29:301–18
16. Cashel, M., Gallant, J. 1974. In *Ribosomes*, ed. M. Nomura, A. Tissieres, C. Fromageot, P. Roeschenthaler, pp. 733–45. Cold Spring Harbor Lab., Cold Spring Harbor
17. Cattaneo, J., Damotte, M., Sigal, N., Sanchez-Medina, G., Puig, J. 1969. *Biochem. Biophys. Res. Commun.* 34: 694–701
18. Cattaneo, J., Sigal, N., Favard, A., Segel, I. H. 1966. *Bull. Soc. Chim. Biol.* 48:441–45
19. Chao, J., Weathersbee, C. J. 1974. *J. Bacteriol.* 117:181–88
20. Chen, G. S., Segel, I. H. 1968. *Arch. Biochem. Biophys.* 127:164–74
21. Chen, G. S., Segel, I. H. 1968. *Arch. Biochem. Biophys.* 127:175–86
22. Cocks, G. T., Wilson, A. C. 1972. *J. Bacteriol.* 110:793–802
23. Cohen, G. N., Stanier, R. Y., Le Bras, G. 1969. *J. Bacteriol.* 68:734–801
24. Cotton, F. A., Hazen, G. G. Jr., Day, V. W., Larsen, S., Norman, J. G., et al. 1973. *J. Am. Chem. Soc.* 95:2367–69
25. Cozzarelli, N. R., Freedburg, W. E., Lin, E. C. C. 1968. *J. Mol.* 31:371–87
26. Crawford, I. P. 1954. *J. Bacteriol.* 68:734–38
27. Crawford, I. P. 1975. *Bacteriol. Rev.* 39:87–120
28. Crawford, I. P., Sikes, S., Melhorn, D. K. 1967. *Arch. Mikrobiol.* 59:72–81
29. Creuzet-Sigal, N., Latil-Damotte, M., Cattaneo, J., Puig, J. 1972. In *Biochemistry of Glycosidic Linkage*, ed. R. Piras, H. G. Pontis, pp. 647–80. New York: Academic
30. Cseke, C., Weeden, N. F., Buchanan, B. B., Uyeda, K. 1982. *Proc. Natl. Acad. Sci. USA* 79:4322–26
31. Damotte, M., Cattaneo, J., Sigal, N., Puig, J. 1968. *Biochem. Biophys. Res. Commun.* 32:916–20
32. Darvill, A. G., Hall, M. A., Fish, J. P., Morris, J. G. 1977. *Can. J. Microbiol.* 23:947–53
33. Dawes, E. A., Senior, P. J. 1973. *Adv. Microbiol. Physiol.* 10:135–266
34. Dietzler, D. N., Leckie, M. P. 1977. *Biochem. Biophys. Res. Commun.* 77: 1459–67
35. Dietzler, D. N., Leckie, M. P., Lais, C. J. 1973. *Arch. Biochem. Biophys.* 156: 684–93
36. Dietzler, D. N., Leckie, M. P., Lais, C. J., Magnani, J. L. 1974. *Arch. Biochem. Biophys.* 162:602–6
37. Dietzler, D. N., Leckie, M. P., Lais, C. J., Magnani, J. L. 1975. *J. Biol. Chem.* 250:2383–87
38. Dietzler, D. N., Leckie, M. P., Lewis, J. W., Porter, S. E., Taxman, T. L., et al. 1979. *J. Biol. Chem.* 254:8295–307
39. Dietzler, D. N., Leckie, M. P., Magnani, J. L., Sughrue, M. J., Bergstein, P. E., et al. 1979. *J. Biol. Chem.* 254:8308–17
40. Dietzler, D. N., Leckie, M. P., Sternheim, W. L., Taxman, T. L., Unger, J. M., et al. 1977. *Biochem. Biophys. Res. Commun.* 77:1468–77
41. Eidels, L., Preiss, J. 1970. *Arch. Biochem. Biophys.* 140:75–89
42. Emeruwa, A. C. 1981. *Ann. Microbiol.* 132B:13–21
43. Ermakova, S., Mansurova, S., Kalebina, T., Lobakova, E., Selyack, I., et al. 1981. *Arch. Microbiol.* 128:394–97
44. Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., et al. 1980. *Science* 209:457–63
45. Fox, J., Kawaguchi, K., Greenberg, E., Preiss, J. 1976. *Biochemistry* 15:849–57
46. Gallant, J. 1979. *Ann. Rev. Genetics* 13:393–415
47. Gentner, N., Greenberg, E., Preiss, J. 1969. *Biochem. Biophys. Res. Commun.* 36:373–80
48. Gibbons, R. J., Kapsimalis, B. 1963. *Arch. Oral Biol.* 8:319–29
49. Govons, S., Gentner, N., Greenberg, E., Preiss, J. 1973. *J. Biol. Chem.* 248: 1731–40
50. Govons, S., Vinopal, R., Ingraham, J., Preiss, J. 1969. *J. Bacteriol.* 97:970–72
51. Greenberg, E., Preiss, J. 1964. *J. Biol. Chem.* 239:4314–15
52. Greenberg, E., Preiss, J. 1981. *J. Bacteriol.* 147:711–19
53. Greenberg, E., Preiss, J. E., van Boldrick, M., Preiss, J. 1983. *Arch. Biochem. Biophys.* 220:594–604
54. Haugen, T. H., Preiss, J. 1979. *J. Biol. Chem.* 254:127–36
55. Haugen, T. H., Ishaque, A., Chatterjee, A. K., Preiss, J. 1974. *FEBS Lett.* 42: 205–8
56. Haugen, T. H., Ishaque, A., Preiss, J. 1976. *J. Biol. Chem.* 251:7880–85
57. Haugen, T. H., Ishaque, A., Preiss, J. 1976. *Biochem. Biophys. Res. Commun.* 69:346–53
58. Haziza, C., Cassan, M., Patte, J.-C. 1982. *Biochimie* 64:227–30
59. Haziza, C., Stragier, P., Patte, J.-C. 1982. *EMBO J.* 1:379–84

60. Hehre, E. J. 1951. *Adv. Enzymol.* 11:297–337
61. Hehre, E. J., Hamilton, D. M. 1946. *J. Biol. Chem.* 166:77–8
62. Hehre, E. J., Hamilton, D. M. 1948. *J. Bacteriol.* 55:197–208
63. Hehre, E. J., Hamilton, D. M., Carlson, A. S. 1949. *J. Biol. Chem.* 177:267–79
64. Hers, H. G., Van Schaftingen, E. 1982. *Biochem. J.* 206:1–12
65. Hestrin, S. 1960. In *The Bacteria*, ed. I. C. Gunsalus, R. Y. Stanier, 3:373–88. New York: Academic
66. Holme, T. 1957. *Acta Chem. Scand.* 11:763–75
67. Holme, T., Palmstierna, H. 1956. *Acta Chem. Scand.* 10:578–86
68. Holmes, E., Boyer, C., Preiss, J. 1982. *J. Bacteriol.* 151:1444–53
69. Huisintveld, J. H. J., Dirks, O. B. 1978. *Caries Res.* 12:243–49
70. Jensen, R. A., Stenmark, S. L. 1970. *J. Bacteriol.* 101:763–69
71. Kamio, Y., Terawaki, Y., Nakajima, T., Matsuda, K. 1981. *Agric. Biol. Chem.* 45:209–16
72. Kappel, W. R., Preiss, J. 1981. *Arch. Biochem. Biophys.* 209:15–28
73. Khandelwal, R. L., Spearman, T. N., Hamilton, I. R. 1973. *Arch. Biochem. Biophys.* 154:295–305
74. Konig, H., Skorko, R., Zillig, W., Reiter, W-D. 1982. *Arch. Microbiol.* 132:297–303
75. Krebs, E. G., Preiss, J. 1975. *MTP Int. Rev. Sci. Carbohydrates, Biochem. Ser. I* 5:337–89
76. Kukko, E., Heinonen, J. 1982. *Eur. J. Biochem.* 127:347–49
77. Lacks, S. 1968. *Genetics* 60:685–706
78. Lahti, R. 1983. *Microbiol. Rev.* 47:169–79
79. Largen, M. T., Belser, W. L. 1975. *J. Bacteriol.* 121:239–49
80. Larsen, C. E., Mukherjee, S., Preiss, J. 1983. *Fed. Proc.* 2193
81. Latil-Damotte, M., Lares, C. 1977. *Mol. Gen. Genetics* 150:325–29
82. Leckie, M. P., Ng, R. H., Porter, S. E., Compton, D. R., Dietzler, D. N. 1983. *J. Biol. Chem.* 258:3813–24
83. Leckie, M. P., Porter, S. E., Tieber, V. L., Dietzler, D. N. 1981. *Biochem. Biophys. Res. Commun.* 99:1433–42
84. Leckie, M. P., Tieber, V. L., Porter, S. E., Deitzler, D. N. 1980. *Biochem. Biophys. Res. Commun.* 95:924–31
85. Lehmann, M., Preiss, J. 1980. *J. Bacteriol.* 143:120–27
86. Levi, C., Preiss, J. 1976. *Plant Physiol.* 58:753–56
87. Lobb, R. R., Stokes, A. M., Hill, H. A. O., Riordan, J. F. 1976. *Eur. J. Biochem.* 70:517–22
88. MacKenzie, C. R., Johnson, K. G., McDonald, I. J. 1977. *Can. J. Microbiol.* 23:1303–7
89. Mackey, B. M., Morris, J. G. 1971. *J. Gen. Microbiol.* 66:1–13
90. Madsen, N. B. 1963. *Can. J. Biochem. Physiol.* 41:561–71
91. Mallette, M. F. 1963. *Ann. NY Acad. Sci.* 102:521–35
92. Marr, A. G., Nilson, E. H., Clark, D. J. 1963. *Ann. NY Acad. Sci.* 102:536–48
93. Maxam, A. M., Gilbert, W. 1980. *Methods Enzymol.* 65:499–560
94. McFarland, C. R., Boyle-Rockwell, P., Rodriguez, J. F. 1981. *Microbios* 32: 143–54
95. McGrew, S. B., Mallette, M. F. 1962. *J. Bacteriol.* 83:844–50
96. McGrew, S. B., Mallette, M. F. 1965. *Nature* 208:1096–97
97. Miernyk, J. A., Dennis, D. T. 1982. *Biochem. Biophys. Res. Commun.* 105: 793–98
98. Mulder, E. G., Deinema, M. H., Van Ween, W. L., Zevenhuizen, L. P. T. M. 1962. *Rec. Trav. Chim. Pays-Bas.* 81: 797–809
99. Murphy, T. M., Mills, S. E. 1969. *J. Bacteriol.* 97:1310–20
100. Nierlich, D. P. 1978. *Ann. Rev. Microbiol.* 32:393–432
101. O'Brien, W. E., Bowen, S., Wood, H. G. 1975. *J. Biol. Chem.* 250:8690–95
102. Okada, G., Hehre, E. J. 1974. *J. Biol. Chem.* 249:126–35
103. Okita, T. W., Rodriguez, R. L., Preiss, J. 1981. *J. Biol. Chem.* 256:6944–52
104. Okita, T. W., Rodriguez, R., Preiss, J. 1982. *Methods Enzymol.* 83:549–53
105. Ouchterlony, O. 1948. *Acta Pathol. Microbiol. Scand.* 25:186–91
106. Palmer, T. N., Wober, G., Whelan, W. J. 1973. *Eur. J. Biochem.* 39:601–12
107. Parsons, T. F., Preiss, J. 1978. *J. Biol. Chem.* 253:6197–202
108. Parsons, T. F., Preiss, J. 1978. *J. Biol. Chem.* 253:7638–45
109. Patel, G. B., Breuil, C. 1981. *Arch. Microbiol.* 129:265–67
110. Pilkis, S. J., El-Maghrabi, M. R., McGrane, M., Pilkis, J., Fox, E., et al. 1982. *Mol. Cell. Endocrinol.* 25:245–66
111. Pirt, S. J. 1965. *Proc. Roy. Soc. London Ser. B* 163:224–31
112. Prager, E. M., Wilson, A. C. 1971. *J. Biol. Chem.* 246:5978–89
113. Preiss, J. 1969. *Curr. Topics Cell Reg.* 1:125–61
114. Preiss, J. 1972. *Intra-Sci. Chem. Reports* 6:13–22
115. Preiss, J. 1973. *The Enzymes* 8:73–119
116. Preiss, J. 1978. In *Advances in Enzymology and Related Areas of Molecular Biology*, ed. A. Meister, 46:317–81. New York: Wiley-Interscience
117. Preiss, J., Greenberg, E. 1983. *Biochem. Biophys. Res. Commun.* 115:820–26
118. Preiss, J., Levi, C. 1980. In *Biochemistry of Plant Carbohydrates: Structure and Function*, ed. J. Preiss, 3:371–423. New York: Academic
119. Preiss, J., Walsh, D. A. 1981. In *Biology of Carbohydrates*, ed. V. Ginsburg, P. Robbins, 1:199–314. New York: Wiley
120. Preiss, J., Crawford, K., Downey, J., Lammel, C., Greenberg, E. 1976. *J. Bacteriol.* 127:193–203
121. Preiss, J., Govons, S., Eidels, L., Lammel, C., Greenberg, E., et al. 1970. *Proc. Miami Winter Symp.*, 1:122–39. Amsterdam: North-Holland
122. Preiss, J., Greenberg, E., Parsons, T. F., Downey, J. 1980. *Arch. Microbiol.* 126: 21–31
123. Preiss, J., Greenberg, E., Sabraw, A. 1975. *J. Biol. Chem.* 250:7631–38
124. Preiss, J., Heubner, J., Greenberg, E. 1982. *Current Microbiol.* 7:257–62
125. Preiss, J., Lammel, C., Greenberg, E. 1976. *Arch. Biochem. Biophys.* 174: 105–19
126. Preiss, J., Mazelis, M., Greenberg, E. 1982. *Current Microbiol.* 7:263–68
127. Preiss, J., Ozbun, J. L., Hawker, J. S., Greenberg, E., Lammel, C. 1973. *Ann. NY Acad. Sci.* 210:265–78
128. Preiss, J., Sabraw, A., Greenberg, E. 1971. *Biochem. Biophys. Res. Commun.* 42:180–96
129. Preiss, J., Shen, L., Greenberg, E., Gentner, N. 1966. *Biochemistry* 5:1833–45
130. Preiss, J., Yung, S-G., Baecker, P. A. 1983. *Mol. Cell. Biochem.* 57:61–80
131. Reeves, R. E. 1968. *J. Biol. Chem.* 243:3202–4
132. Reeves, R. E., South, D. J., Blyth, H. J., Warren, L. G. 1974. *J. Biol. Chem.* 249:7737–74
133. Ribbons, D. W., Dawes, E. A. 1963. *Ann. NY Acad. Sci.* 102:564–86
134. Ribereau-Gayon, G., Sabraw, A., Lammel, C., Preiss, J. 1971. *Arch. Biochem. Biophys.* 142:675–92
135. Richaud, F., Richaud, C., Haziza, C., Patte, J.-C. 1981. *C. R. Acad. Sci. Ser. III* 293:507–12
136. Riordan, J. F., Elvany, K. D., Borders, C. L. Jr. 1977. *Science* 195:884–86
137. Robakis, N., Maza-Basso, L., Brot, N., Weissbach, H. 1981. *Proc. Natl. Acad. Sci. USA* 78:4261–64
138. Rocha, V., Crawford, I. P., Mills, S. E. 1969. *J. Bacteriol.* 111:163–68
139. Rohlfing, S. R., Crawford, I. P. 1966. *J. Bacteriol.* 91:1085–97
140. Sabularse, D. C., Anderson, R. L. 1981. *Biochem. Biophys. Res. Commun.* 100: 1423–29
141. Sabularse, D. C., Anderson, R. L. 1981. *Biochem. Biophys. Res. Commun.* 103: 848–55
142. Sarma, T. A., Kanta, S. 1979. *Z. Allg. Mikrobiol.* 19:571–75
143. Schwartz, M. 1965. *C. R. Acad. Sci.* 260:2613–16
144. Schwartz, M. 1966. *J. Bacteriol.* 92: 1083–89
145. Segel, I. H., Cattaneo, J., Sigal, N. 1965. *Proc. Int. CNRS Symp. Mech. Regulation Cellular Activities of Microorganisms, Marseilles 1963*, pp. 337–55. Paris: Ctr. Natl. Rech. Sci.
146. Shaw, D. H., Squires, M. J. 1980. *Arch. Microbiol.* 125:83–87
147. Shen, L. C., Atkinson, D. E. 1970. *J. Biol. Chem.* 245:3996–4000
148. Shen, L., Preiss, J. 1965. *J. Biol. Chem.* 240:2334–40
149. Shen, L., Ghosh, H. P., Greenberg, E., Preiss, J. 1964. *Biochim. Biophys. Acta* 89:370–72
150. Shine, J., Dalgarno, L. 1974. *Proc. Natl. Acad. Sci. USA* 71:1342–46
151. Sigal, N., Puig, J. 1968. *C. R. Acad. Sci.* 267:1223–26
152. Sigal, N., Cattaneo, J., Chambost, J. P., Favard, A. 1965. *Biochem. Biophys. Res. Commun.* 20:616–20
153. Sigal, N., Cattaneo, J., Segel, I. H. 1964. *Arch. Biochem. Biophys.* 108: 440–51
154. Slock, J. A., Stahly, D. P. 1974. *J. Bacteriol.* 120:399–406
155. Smith, A. J. 1975. *Biochem. Soc. Trans.* 3:12–14
156. Stanier, R. Y., Adams, G. A. 1944. *Biochem. J.* 38:168–71
157. Steigerwalt, A. G., Fanning, G. R., Fife-Asbury, M. A., Brenner, D. J. 1976. *Can. J. Microbiol.* 22:121–37
158. Steiner, K. E., Preiss, J. 1977. *J. Bacteriol.* 129:246–53
159. Strange, R. E. 1968. *Nature* 220:606–7
160. Strange, R. E., Dark, F. A., Ness, A. G. 1961. *J. Gen. Microbiol.* 25:61–76
161. Strasdine, G. A. 1968. *Can. J. Microbiol.* 14:1059–62
162. Strasdine, G. A. 1972. *Can. J. Microbiol.* 18:211–17
163. Taguchi, M., Izui, K., Katsuki, H. 1980. *J. Biochem. (Japan)* 88:379–87
164. Tanzer, J. M., Freedman, M. L., Woodiel, F. N., Eifert, R. L., Rineheimer, L. A. 1976. *J. Dental Res.* 55B:173
165. Tempest, D. W., Strange, R. E. 1966. *J. Gen. Microbiol.* 44:273–79
166. Urbanowski, J., Leung, P., Weissbach, H., Preiss, J. 1983. *J. Biol. Chem.* 258:2782–84

167. Uyeda, K., Furuya, E., Sherry, A. D. 1981. *J. Biol. Chem.* 256:8679–84
168. Van Houte, J., Jansen, H. M. 1970. *J. Bacteriol.* 101:1083–85
169. Van Houte, J., Saxton, C. A. 1971. *Caries Res.* 5:30–43
170. Walker, G. J. 1966. *Biochem. J.* 101:861–72
171. Wilkinson, J. F. 1959. *Exptl. Cell Res. Suppl.* 7:111–30
172. Wober, G. 1973. *Z. Physiol. Chem.* 354:75–82
173. Yanofsky, C., Platt, T., Crawford, I. P., Nichols, B. P., Christie, G. E., et al. 1981. *Nucleic Acids Res.* 9:6647–68
174. Yung, S.-G., Preiss, J. 1981. *J. Bacteriol.* 147:101–9
175. Yung, S.-G., Preiss, J. 1982. *J. Bacteriol.* 151:742–49
176. Yung, S.-G., Paule, M., Beggs, R., Greenberg, E., Preiss, J. 1984. *Arch. Microbiol.* 138:1–8
177. Zevenhuizen, L. P. T. M. 1964. *Biochim. Biophys. Acta* 81:608–11
178. Zevenhuizen, L. P. T. M. 1966. *J. Microbiol. Serol.* 32:356–72
179. Zevenhuizen, L. P. T. M. 1981. *Antonie van Leeuwenhoek* 47:481–97

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THE STATUS OF Y_{ATP} AND MAINTENANCE ENERGY AS BIOLOGICALLY INTERPRETABLE PHENOMENA

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INTRODUCTION

The capacity to grow, and ultimately to multiply, is one of the most fundamental characteristics of living cells. Indeed, with microbial cells this often is the sole criterion used to assess whether or not such creatures are alive. Yet despite the fact that growth is such a basic aspect of microbial behavior, little attention seemingly was paid to the underlying principles prior to the classic studies of Monod (34) and the advent of continuous culture techniques (35, 42). Previously, it generally was considered sufficient to record growth as being either evident (+) or not (0), with occasional recourse to semiquantitative variations such as +++ (confluent) and ± (scant). And whereas these essentially qualitative assessments often proved adequate, there can be little doubt

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that the more quantitative approaches that have been adopted over recent decades have provided a vastly more penetrating insight into the nature of microbial life.

However, interpretations of these quantitative data in physiological terms, as well as their structuring in mathematical equations (nowadays popularly called "models"), have not invariably met with consensus; and nowhere is this more evident than in studies of microbial growth energetics (15, 58). Here, it is clear that although knowledge of the mechanisms by which living cells generate biologically useful energy has advanced apace, an understanding of how energy fluxes are regulated in relation to the needs of cell synthesis still is largely lacking. Indeed, the early promise that such an understanding could be derived from analyses of microbial growth yield data has not been fulfilled. Nevertheless, these data have served to identify some important aspects of microbial behavior that are relevant in both a physiological and an industrial context.

It would seem appropriate, therefore, to look afresh at the basic concepts, to try to identify the sources of controversy, and, in particular, to evaluate the current status of Y_{ATP} and "maintenance energy" as biologically interpretable phenomena.

In the above connection, it is also clear that the energy required to fuel biosynthesis can be derived from a number of sources: from light, or from the oxidation of a variety of inorganic and organic compounds. However, because the bulk of data on microbial growth energetics currently available comes from studies of heterotrophic bacteria, we shall concentrate on these organisms, although the same energetic considerations well might apply equally to the growth of phototrophic and chemolithotrophic bacteria.

The Concept of Y_{ATP}

As intimated above, the foundation for studies of microbial growth energetics was laid by the finding (34) of a stoichiometric relationship between growth and carbon substrate consumption. Here, studies of *Escherichia coli, Salmonella typhimurium,* and *Bacillus subtilis,* growing in batch culture on a variety of polyalcohols, hexoses, pentoses, methylpentoses, and di- and polysaccharides showed that the yield (mg organisms formed/mg carbon substrate consumed) was, within limits, independent of the initial substrate concentration. Moreover, a close proportionality was observed between the increase in cell density (x) and the decrease in substrate concentration (s), indicating that the yield value was constant throughout the exponential growth phase; that is, that there was a direct relationship between the specific growth rate ($\mu = 1/x \cdot dx/dt$) and the specific rate of substrate consumption ($q = -1/x \cdot ds/dt$). These cultures were grown aerobically in a simple mineral medium, and it is of particular interest that, for a given carbohydrate, the three organisms expressed approximately the same dry weight yields.

In contrast to these early studies of aerobically growing cultures, in which the carbon substrate served as both a source of energy and of cell carbon, Gunsalus and his colleagues (6, 10) studied the growth of *Streptococcus faecalis* and *Leuconostoc mesenteroides* in a complex medium that provided the bulk of the cell carbon, and in which the energy requirements were met by the anaerobic catabolism of a single added carbohydrate. Here, carbon substrate (glucose) was converted almost quantitatively either to lactic acid (by *S. faecalis*) or to lactic acid, ethanol, and CO_2 (by *L. mesenteroides*), and, significantly, the yield of *S. faecalis* (per mol of glucose fermented) was substantially higher than that of *L. mesenteroides*. They concluded from these results that, because both organisms used glucose mainly as an energy source, and because growth was proportional to the amount of energy source supplied, *S. faecalis* obtained more energy per mol of glucose than did *L. mesenteroides*, and that the latter organism fermented glucose by a mechanism that did not involve the Embden-Meyerhof pathway.

This conclusion was of considerable importance because it was later shown (21) that not only did *L. mesenteroides* ferment glucose by a hitherto unknown pathway involving xylulose 5-phosphate as a key intermediate (13), but also that this pathway yielded net but 1 mol ATP per mol glucose fermented compared with 2 mol ATP generated net by the reactions of the Embden-Meyerhof pathway. Moreover, from these results it further appeared that growth could be stoichiometrically related to the amount of ATP produced.

In order to study in more detail the relationship between growth and energy supply, Elsden and his colleagues (1, 50) measured the weight of organisms produced when *S. faecalis* was supplied with glucose, ribose, and L-arginine (separately) as energy sources, and when *Saccharomyces cerevisiae* and *Pseudomonas lindneri* (now named *Zymomonas mobilis*) were supplied with glucose. The latter two organisms were selected because both fermented glucose to ethanol and CO_2 but by different pathways that were energetically different (in terms of net ATP gain).

Finally, they measured the growth of *Propionibacterium pentosaceum* on glucose, glycerol, and DL-lactate. From these extensive studies they were able to conclude that there was a linear relationship between the dry weight of organisms produced and the concentration of energy source in the medium. Thus arose the concept of an approximately constant Y_{ATP} value for microbial growth of 10.5 (g cells formed per mol ATP generated by substrate catabolism). However, as the authors of this concept were careful to point out (11, 50), unequivocal evaluations of growth in terms of ATP flux could be made only with cultures in which (a) insignificant amounts of energy source carbon were assimilated into cell substance, (b) the quantity of energy-yielding substrate consumed, and the net ATP gain, were known with certainty, and (c) growth was limited by the supply of energy source. In this latter connection, they were aware of the fact that "obligatory coupling of glycolysis, or respira-

tion, and cell growth is not universal" (11) and hence a maximum degree of coupling only could be assumed when growth was energy-limited.

The Concept of Maintenance

Much of the early work on growth yields was performed using batch cultures in which organisms grow at their maximum rate in an environment that, as a consequence of metabolism, changes progressively with time. And whereas the yield data obtained were highly reproducible, it was recognized that interpretation of these data inevitably demanded the assumption that growth-associated changes in environment exerted only a negligible effect on the overall conversion of substrate to biomass. More satisfactory, it was argued (11, 50), was the use of chemostat culture techniques, because these allowed organisms to be grown at a variety of predetermined submaximal rates in environments that no longer varied with time. Under these so-called steady-state conditions, the yield values could be readily derived from the ratio of the specific growth rate (numerically equal to the dilution rate, D = medium flow rate divided by the culture volume) and the specific rate of substrate consumption (i.e. $Y = \mu/q = D/q$).

It followed, therefore, that if the yield was a true constant, then q should vary linearly with the dilution rate and, on extrapolation, decline to zero at zero dilution rate. This was quickly found not to be the case with most aerobic and anaerobic carbon substrate limited cultures (16, 18); and although the relationship between q and D generally was more or less linear, on extrapolation to zero dilution rate q tended to some finite value (Figure 1a). In other words, as the growth rate of these aerobic cultures was decreased, more of the influent carbon substrate was catabolized to product (CO_2) and less was assimilated into cell substance (Figure 1b); thus the yield value declined towards zero.

In order to explain these findings, it was initially suggested (16) that, in addition to the anabolic metabolism of organisms, they express a constant endogenous metabolism by which *cell substance* is oxidized to CO_2. Mathematically, this could be expressed by inserting into the specific growth rate equation a constant term (k) representing the endogenous metabolism. Thus

$$dx/dt = (\mu - k)x, \qquad 1.$$

where k has the same dimensions as growth rate and was later replaced by the term μ_e (49). However, though growing cells well may express an endogenous metabolism [e.g. turn over their macromolecular components such as proteins (45) and peptidoglycan (5)], it was equally plausible to assume that some of the carbon substrate consumed and not assimilated into cell substance served to generate energy required to fulfil functions other than growth (e.g. maintenance of solute gradients, osmotic regulation, motility). Therefore it could be argued that one might better represent mathematically the relationship between growth rate and yield value in terms of substrate being consumed to fulfil a

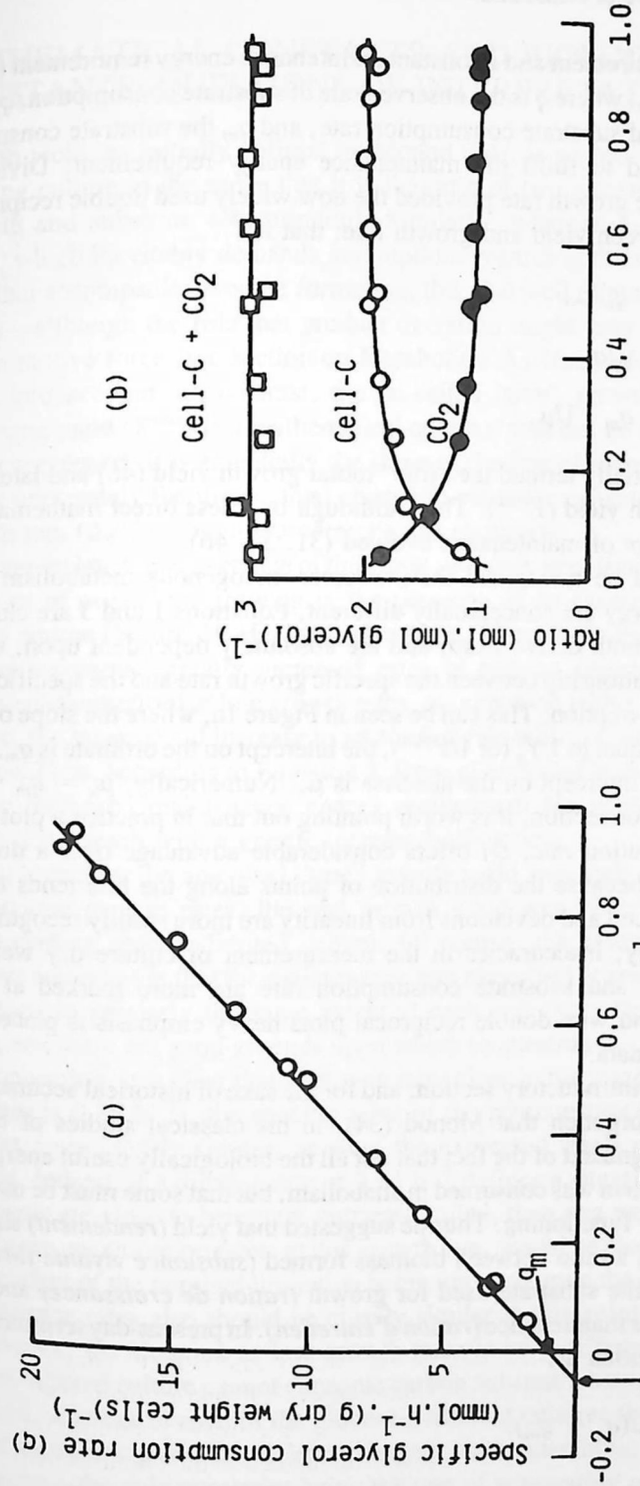


Figure 1 Influence of dilution rate on (*a*) the specific rate of glycerol consumption and (*b*) the distribution of substrate carbon between cells and CO_2; observed with a glycerol-limited chemostat culture of *Klebsiella aerogenes* NCTC 418 growing in a mineral medium at 37°C (see Ref. 18).

fixed growth requirement and a constant maintenance energy requirement (46). Thus $q = q_g + q_m$, where q is the observed rate of substrate consumption, q_g the growth-associated substrate consumption rate, and q_m the substrate consumption rate required to fulfil the maintenance energy requirement. Dividing throughout by the growth rate provided the now widely used double reciprocal relationship between yield and growth rate; that is,

$$q/\mu = q_g/\mu + q_m/\mu, \qquad 2.$$

or, because $Y = \mu/q$,

$$1/Y = 1/Y_g + q_m \cdot 1/\mu, \qquad 3.$$

where Y_g was initially termed the "true" molar growth yield (46) and later the maximum growth yield (Y^{max}). Thus, although by a less direct mathematical route, the concept of maintenance evolved (31, 32, 46).

Here it should be mentioned that, whereas endogenous metabolism and maintenance energy are conceptually different, Equations 1 and 3 are closely related. In fact, both derive from, and are absolutely dependent upon, there being a linear relationship between the specific growth rate and the specific rate of substrate consumption. This can be seen in Figure 1a, where the slope of the line ($\Delta q/\Delta\mu$) is equal to $1/Y_g$ (or $1/Y^{max}$), the intercept on the ordinate is q_m, and the extrapolated intercept on the abscissa is μ_e. Numerically, $\mu_e = q_m \cdot Y_g$.

In the above connection, it is worth pointing out that in practice a plot of q versus μ (or dilution rate, D) offers considerable advantage over a double reciprocal plot, because the distribution of points along the line tends to be more evenly spaced and deviations from linearity are more readily recognized. More importantly, inaccuracies in the measurement of culture dry weight, respiration rate, and substrate consumption rate are more marked at low dilution rates, and with double reciprocal plots heavy emphasis is placed on these particular data.

Finally in this introductory section, and for the sake of historical accuracy, it should not be forgotten that Monod (34), in his classical studies of batch cultures, was cognizant of the fact that not all the biologically useful energy of substrate catabolism was consumed in anabolism, but that some must be used to maintain cellular functioning. Thus he suggested that yield *(rendement)* should be considered as a ratio between biomass formed *(substance vivante formée)* and the sum of the substrate used for growth *(ration de croissance)* and the substrate used for maintenance *(ration d'entretien)*. In present-day terminology this could be written as:

$$Y = \mu/q = \mu/(q_g + q_m),$$

which is, of course, the reciprocal of Equation 2.

MATHEMATICAL CONSTANTS AND BIOLOGICAL CONSTANTS: QUESTIONS OF INTERPRETATION

Growth yield, as initially defined by Monod (34), is a "real" parameter of a growing culture in the sense that it is the ratio of two measurable quantities (growth and substrate consumption). Similarly, whereas Y_{ATP} is a derived value, which inevitably demands assumptions regarding the net formation of ATP that accompanies product formation, this also well might approximate to reality—although the role that product excretion might play in generating a proton motive force (see section on Metabolites As Uncouplers) needs to be taken into account. In contrast, the so-called "true" growth yield (Y_g) or maximum yield (Y^{max}) is a mathematical constant that has no direct physiological counterpart. It is essentially the slope of the line of regression of q on μ and defines solely the incremental change in substrate consumption rate with growth rate ($\Delta q/\Delta\mu$) or, more correctly, its reciprocal.

To appreciate the importance of this point, it is only necessary to examine the behavior of organisms growing in the presence of an excess of carbon and energy source (Figure 2). Here it is clear that, whereas the glucose-sufficient cultures expressed greatly increased rates of oxygen consumption (and of glucose consumption) at low growth rates, as compared with a glucose-limited culture, the incremental increase in respiration rate with growth rate was much smaller. Thus, according to Equation 3, these glucose-sufficient cultures had a greatly increased maintenance energy requirement but used the remaining energy for growth with a greatly increased efficiency.

Paradoxically, the so-called "true" growth yield was high, whereas, at all submaximal growth rates, the real growth yield was lower than that of a corresponding glucose-limited culture. Of course, the assumption that this analysis embodies is that the maintenance rate expressed at zero growth rate is expressed to precisely the same extent at each and every finite growth-rate value; and there are good grounds upon which to question this assumption. In this connection, it is clear that at growth rates close to the maximum (which in this case is 0.85 h^{-1}), the specific rates of oxygen consumption of the four cultures were closely similar, as were the expressed (real) yield values for oxygen. Indeed, if a dilution rate of 0.85 h^{-1} defines a situation in which the organisms are close to being not nutrient-limited, then one would expect that the oxygen consumption rates, at this growth rate, would be closely similar and independent of the nominal limitation being imposed upon the culture; and the maintenance rates also should be closely similar at this point.

However, the situation at low growth rates is very different, because the glucose-limited culture cannot consume carbon substrate faster than it is being supplied, whereas in each of the glucose-sufficient cultures the rate of oxidation of the excess glucose will depend largely on the metabolic potential of the organisms—the only constraint being the rate of penetration of substrate into

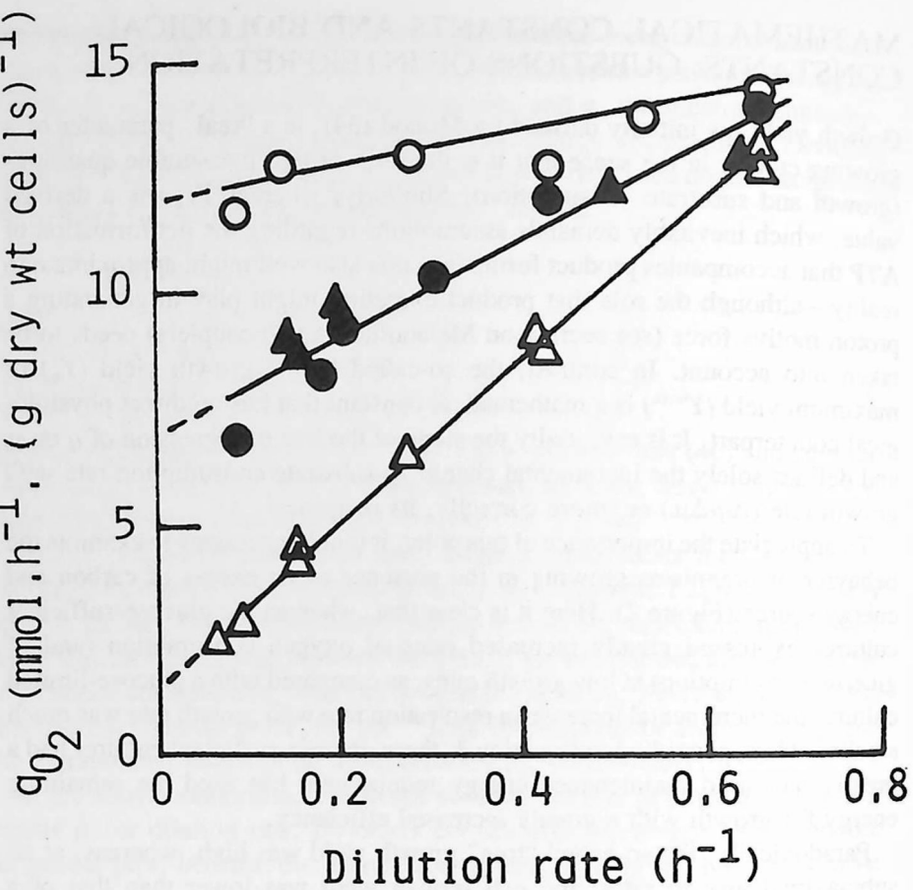


Figure 2 Influence of dilution rate on the specific respiration rates of chemostat cultures of *Klebsiella aerogenes* NCTC 418 growing in a mineral medium plus glucose; (Δ) glucose-limited, (▲) ammonia-limited, (●) sulphate-limited, and (○) phosphate-limited (see Ref. 39).

the cells and possible feedback regulation of glycolysis and respiration. From Figure 2 it is obvious that, at low growth rates, the oxidative potential of glucose-sufficient organisms is vastly greater than that which would be required to meet the cells' minimum biosynthetic and bioenergetic demands (as indicated by the rate of respiration of the glucose-limited culture). But if, at growth rates close to μ_{max}, the organisms were growing with a similar efficiency, then clearly the amount of oxygen consumed in processes not associated with growth must be greatly decreased over that extant at low growth rates. That is to say, the maintenance rate of oxygen consumption must decrease progressively as the growth rate is increased. If this is indeed the case, then equally clearly the $Y_{O_2}^{max}$ value derived from Equation 3 must be grossly overestimated.

It is, of course, possible to modify Equation 3 to accommodate a growth rate–linked change in "maintenance." Thus, making the sole assumption that if

the maintenance rate varies it does so in a linear fashion, one can define the relationship as follows:

$$1/Y^{max} = (q - q_m\,(1 \pm c\mu))/\mu,$$

where q_m again denotes the specific rate of substrate consumption at zero growth rate, and where "c" is a constant that defines the slope of the line of variation of maintenance rate with growth rate ($\Delta q_m/\Delta\mu$). Thus

$$1/Y = (1/Y^{max} \pm c \cdot q_m) + q_m/\mu. \qquad 4.$$

Equation 4 was initially formulated (40) to incorporate the possibility that growth-unassociated energy dissipation may proceed at different rates in faster growing cultures as compared with slower growing ones, and it was recognized that this formulation did not lead to a more precise evaluation of Y^{max} because no numerical value could be placed on the constant "c." All it was intended to show was that the values of Y^{max} derived from Equation 3, although mathematically correct, could not be accepted as true biological constants. This idea was subsequently reworked into a number of similar (mathematically identical) formulations (22, 47, 52, 69), which suggest that there are two components to maintenance: one that is growth-rate dependent and one that is constant (growth-rate independent). And whereas one cannot exclude the possibility that some components of the total maintenance energy requirement do not vary with growth rate, there are no good physiological grounds for interpreting Equation 4 in that way. Clearly what is needed is a better understanding of what, precisely, constitutes "maintenance"; this we consider in the next section.

Maintenance Functions

Whereas microorganisms possess the capacity to turn over many of their macromolecular components, like RNA, protein, and cell-wall polymers (5, 45), the rates at which these are found to proceed generally are sufficient to account for only a small part of the total energy dissipated in growth-unassociated functions. Therefore it is necessary to consider other processes that might contribute substantially to the overall maintenance energy requirement and, in particular, to seek to understand how energy is being dissipated in those carbon substrate–sufficient cultures where catabolism is extensively dissociated from anabolism (Figure 2).

ION GRADIENTS On thermodynamic grounds one might conclude that, among the many growth-unassociated functions that demand an expenditure of energy, is the maintenance of solute gradients. In gram-negative bacteria like *Klebsiella aerogenes*, the principal low-molecular-weight solutes present intracellularly are K^+ and glutamic acid (57, 60), whose concentrations vary markedly with growth rate (57) and with the osmolarity of the medium (8, 59).

Moreover, the K^+ is exceedingly mobile and rapidly fluxes from the cell when respiration rate is impeded (19).

Studies of the behavior of *K. aerogenes* in glucose-limited chemostat culture (19) revealed that increasing or decreasing the extracellular K^+ concentration (and thereby changing the magnitude of the transmembrane K^+ gradient) elicited marked changes in the steady state respiration rate (Figure 3a), a finding consistent with the conclusion that maintenance of a transmembrane K^+ gradient is energetically expensive. Moreover, this conclusion was further supported by a finding of a more or less linear relationship between the respiration rate and the electrochemical equivalent of the transmembrane K^+ gradient, as calculated by the Nernst equation (Figure 3b).

Of significance, here, is the fact that the cellular K^+ content of *K. aerogenes* has been found to vary progressively with the growth rate, irrespective of the nature of the growth limitation (57); it follows, therefore, that so too must the magnitude of the transmembrane K^+ gradient. Therefore, if maintenance of this gradient accounts for a substantial proportion of the total energy expended in growth-unassociated functions, then one might expect the overall maintenance requirement to vary with growth rate. This has been tested by growing *K. aerogenes* in a glucose-limited chemostat culture that contained, respectively, a 10-fold excess of K^+ (i.e. 10 mM) and an amount of K^+ (1 mM) just sufficient to meet the cellular requirement of a rapidly growing culture. Varying the dilution rate effected changes in respiration rate as shown in Figure 4a. Because the population density increased to some extent with growth rate (as generally is found with carbon substrate–limited cultures), and because the cellular K^+ requirement increased substantially (from 0.81%, w/w, of the dry weight at $D = 0.1\ h^{-1}$, to 1.57% at $D = 0.75\ h^{-1}$), the extracellular K^+ concentration diminished as both the growth rate and the intracellular K^+ concentration increased, the effect being more marked with the low-K^+ culture than with the high-K^+ culture (Figure 4b).

Now one can deduce from Figure 3b that an increase in transmembrane K^+ gradient equivalent to 10 mV needs to be supported by an increased respiration rate of 0.28 mmol $O_2 \cdot h^{-1} \cdot (\text{mg dry weight cells})^{-1}$, and one can therefore calculate the changes in this maintenance energy rate (i.e. oxygen consumption supporting the K^+ gradient) with growth rate for the two cultures. Deducting this rate from the corresponding rate of respiration at each growth rate gives corrected respiration rates that again vary linearly with growth rate but significantly extrapolate to the origin (Figure 4a). Thus one might conclude that more than 90% of the maintenance energy requirement of these glucose-limited *K. aerogenes* cultures served to support and maintain the transmembrane K^+ gradient. Moreover, if this is the case, then it is obvious that the maintenance energy requirement must indeed vary with growth rate even in carbon substrate–limited cultures containing a 10-fold excess of K^+.

Because medium salinity also influences the cellular K^+ content (8, 59), one

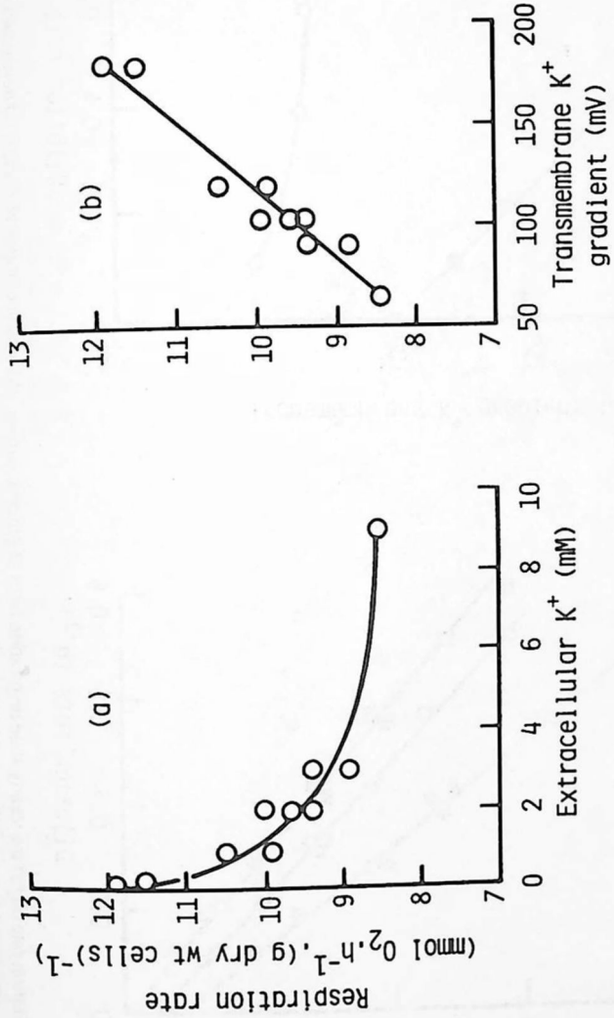


Figure 3 Influence of changes in the extracellular K^+ concentration on (*a*) the steady state respiration rate, and (*b*) the transmembrane K^+ gradient of *Klebsiella aerogenes* NCTC 418 growing in a glucose-limited chemostat culture at a dilution rate of 0.4 h^{-1} (35°C, pH 6.8) (see Ref. 19).

might expect this to affect concomitantly the maintenance energy requirement; such was found to be the case with yeast cultures (68), although similar studies with *K. aerogenes* gave equivocal results (36). Addition of 2%, w/v, NaCl to a carbon-limited *K. aerogenes* culture caused the cellular K^+ content to increase two-fold [from 1.3% of the dry weight to 2.5% at a dilution rate of 0.3 h^{-1} (59)]; assuming a similar proportional increase at all dilution rates, one can calculate the expected effect of the presence of 2%, w/v, NaCl in the environment on the respiration rate of a glucose-limited culture containing 10 mM K^+ in the influent medium. The data thus obtained reveal that, although the effect should be significant, it would be insufficient to be readily detectable experimentally.

The apparent high requirement for metabolic energy associated with maintenance of a transmembrane K^+ gradient can be rationalized in terms of a K^+ leakage current whose magnitude varies with the size of the gradient. This, then, leads to a consideration of whether energy also might be dissipated as a consequence of *proton* leakage (15). Here it has been shown (37) that addition of a protonophorous uncoupler, 2,4-dinitrophenol (DNP), to a growing glucose-limited chemostat culture greatly increased the steady-state respiration rate (i.e. the maintenance energy requirement) at all growth rates; but whether, and to what extent, proton leakage occurs naturally remains to be established. Of particular relevance is the behavior of glucose-sufficient chemostat cultures in which a high respiration rate is expressed at low growth rates (Figure 2). For whereas one might plausibly argue that, with a K^+-limited culture, energy was being dissipated at the level of the membrane, this is not so clearly the case with other glucose-sufficient cultures (see also 2, 7, 54). Hence a question arises as to the possible mechanism(s) by which, say, ammonia- or sulphate-limited cultures dispose of the excess respiratory energy that is generated, surplus to their biosynthetic needs. Here one can envisage a number of possibilities, besides the direct involvement of an ATPase, such as (*a*) futile cycles, (*b*) metabolites acting as uncouplers, (*c*) modification of respiratory chain components and/or activities, (*d*) mechanisms that bypass energy-conserving loops on the respiratory chain, and (*e*) mechanisms that bypass substrate-level phosphorylation steps and thereby metabolically uncouple glycolysis.

FUTILE CYCLES When two compounds can be interconverted by reactions that are irreversible, and the enzymes catalyzing these reactions are both active,then there is "futile cycling" in which dissipation of energy occurs without a corresponding change in metabolites (25). For example, there are three irreversible steps in the interconversion of glucose to pyruvate: (*a*) between glucose and glucose 6-phosphate, (*b*) between fructose 6-phosphate and fructose 1,6-bisphosphate, and (*c*) between pyruvate and phospho*enol*-pyruvate. With mammalian systems studied in vitro, cycling has been observed at each of these steps. Similarly, the futile cycle associated with phosphofructo-

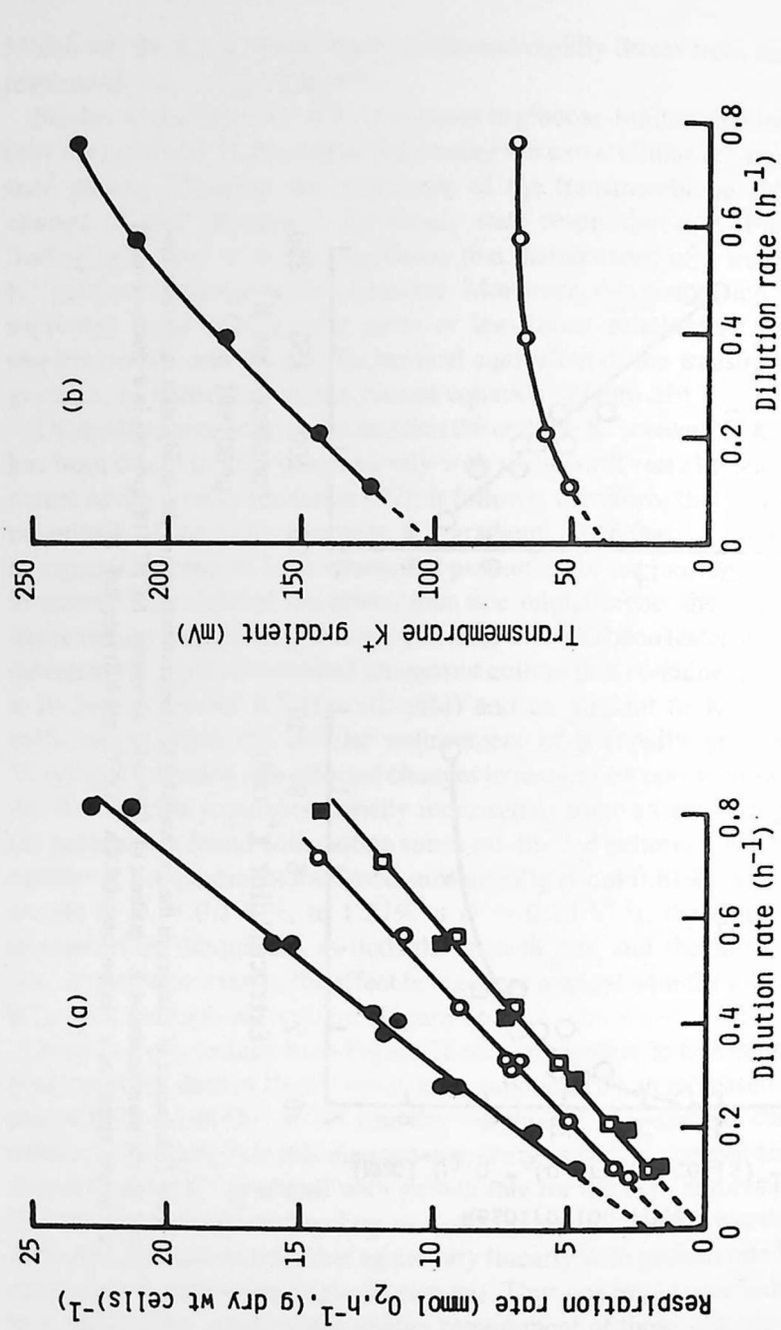


Figure 4 Influence of dilution rate on (*a*) the steady state respiration rates of glucose-limited chemostat cultures of *Klebsiella aerogenes* NCTC 418 growing in mineral media containing (●) 1 mM K^+ and (○) 10 mM K^+. In (*b*) are plotted the respective steady-state transmembrane K^+ gradients extant at each dilution rate for the two cultures. From the magnitude of these gradients, at each dilution rate, the appropriate rate of oxygen consumption required for maintenance (of the K^+ gradient) was estimated, and this value subtracted from the overall rate of oxygen consumption in situ to give the data points (■, □) contained in (*a*).

kinase (PFK) and fructose 1,6-bisphosphatase (FBPase) has been detected in *E. coli* and its activity assessed quantitatively (4). The net effect of these reactions is to hydrolyze ATP, as illustrated by Figure 5.

$$\text{Fructose 6-phosphate} + \text{ATP} \xrightarrow{\text{PFK}} \text{Fructose 1,6-P}_2 + \text{ADP}$$

$$\text{Fructose 1,6-P}_2 \xrightarrow{\text{FBPase}} \text{Fructose 6-phosphate} + \text{Pi}$$

$$\text{Sum:} \qquad \text{ATP} \longrightarrow \text{ADP} + \text{Pi}$$

Figure 5 Hydrolysis of ATP by means of a futile cycle.

Under conditions of ammonia limitation, organisms like *K. aerogenes* are found to contain high levels of glutamine synthetase and significant levels of glutaminase that, together, also can effect ATP hydrolysis, as was suggested by Gunsalus & Shuster (11). Clearly, in the growing cell the activities of the relevant enzymes must be highly regulated; but how this is effected and, indeed, why some futile cycles are present is not fully understood.

A possible explanation for the presence in ammonia-limited cells of glutaminase, which acts upon the primary product of ammonia assimilation (glutamine), is that it allows the key enzyme of ammonia assimilation (glutamine synthetase) to continue working at a significant rate in the virtual absence of exogenous ammonia. This, one might argue, permits the uptake system to remain in a highly reactive state with respect to transient changes in the supply of growth-limiting nutrient.

If, as suggested, some futile cycles serve as "sensors" for the uptake of growth-limiting nutrients, one might expect similar reactions to be associated with common nutrients such as sulphate and phosphate; indeed, it has been shown (64) that, when growing on sulphate, *E. coli* possesses two enzymes (adenosine5'-phosphosulphate kinase and 3'(2'),5'-diphosphonucleoside 3'-phosphohydrolase) that are potentially capable of acting in concert as a futile cycle. Moreover, it has been long established that phosphatases are synthesized to high levels by phosphate-limited cells (63); and although these enzymes may be secreted in substantial amounts, frequently they are retained in the cytoplasm and cell envelope (71).

Because ATP is the primary product of phosphate assimilation and is an intermediate in nucleic acid synthesis, it is logical that ATP turnover should occur by way of a futile cycle when the supply of phosphate is interrupted. Clearly, if no cycling occurred in the absence of exogenous phosphate, then the adenine nucleotide pool would rapidly become so depleted as to render the cells totally unreactive and probably nonviable. Indeed, the presence of an active

futile cycle in phosphate-limited cells was indicated by the fact that the respiration rate of a phosphate-limited *K. aerogenes* culture (growing at $D = 0.1\ h^{-1}$) did not diminish over an extended period of time (20 min) when the exogenous supply of phosphate was withdrawn (36, 61).

Thus, since no respiratory control was evident, these cells presumably were able either to maintain an adequate pool of ADP and Pi or else (as seems less likely) to totally dissociate respiration from ADP phosphorylation. In this connection, it is relevant to mention that to sustain active growth even at this low rate ($0.1\ h^{-1}$), organisms like *K. aerogenes* would need to consume phosphate at a rate of about $0.8\ \mu mol \cdot min^{-1} \cdot (g\ dry\ wt\ cells)^{-1}$, whereas the estimated steady-state extracellular phosphate concentration would be substantially less than 0.4 μM (the apparent K_s of the uptake system). Hence, with a steady-state phosphate-limited culture containing $1\ g \cdot liter^{-1}$ dry weight organisms, the extracellular phosphate concentration would be sufficient to sustain growth at the same rate for maximally 30 sec, without replenishment.

Though potential futile cycles can be readily identified, it is not easy to demonstrate their actual functioning in growing cells, nor to quantify them (but see 4), and the same is true for membrane-associated energy-spilling reactions. However, again, evidence for their existence is less equivocal.

METABOLITES AS UNCOUPLERS It has been known for many years that weak organic acids can traverse the cytoplasmic membrane when present in the undissociated form (26, 48); and some of these acids (like acetic and propionic acids) are major products of carbon substrate catabolism. Thus, because the concentration of undissociated acid in solution depends critically on the pH value, according to the Henderson-Hasselbalch equation

$$\log \frac{(A^-)}{HA} = pH - pK_a,$$

high extracellular concentrations of such acids can act as uncouplers when the medium pH value is substantially less than that of the cytoplasm. This uncoupling is, of course, due to the passive diffusion of the undissociated acid into the cytoplasm and its subsequent ionization (at the higher cytoplasmic pH value). The net effect, in the absence of metabolic activity, would be to equalize the intra- and extracellular pH values and thus eliminate the ΔpH component of the proton motive force (Figure 6a). However, just as with artificial protonophorous uncouplers like DNP, organisms can overcome this effect by increasing their respiration rate (20), thereby pumping protons from the cytoplasm at an increased rate (Figure 6b).

In the experiment depicted in this latter figure, the yeast *Candida utilis* was first grown in an acetate-limited chemostat culture at pH values above and below the growth optimum (pH 5.5), and the steady-state respiration rates were

determined. Whereas pH had some effect on the growth efficiency (i.e. the respiration rates required to sustain growth at a fixed rate varied significantly), steady-state conditions could be established at pH values between 7.0 and 2.5.

In contrast, an ammonia-limited culture, growing in the presence of excess acetate, was exceedingly sensitive to the extracellular (controlled) pH value. As the pH was progressively lowered from 7.0, respiration increased sharply to a peak rate at pH 5.2; as the pH was decreased further, respiration collapsed and the culture washed out from the fermenter. Presumably, at pH values below 5.0, the organisms were no longer able to pump protons from the cytoplasm at a rate equal to that at which the undissociated acid was entering the cell. Thus, the cytoplasm became acidified, thereby inhibiting metabolism and the supply of reductant to the respiratory chain.

Organic acids like acetate seemingly exert a lesser influence on the growth of some bacteria (e.g. *K. aerogenes*), and, indeed, evidence has been presented (33, 62) that efflux of lactate from *Streptococcus cremoris* cells, down a concentration gradient, actually can serve to generate a membrane potential when these organisms are cultured anaerobically on lactose at relatively high pH values (pH 6.8). The calculated energy gain from lactate efflux was estimated to be almost 2 ATP equivalents per mol of lactose fermented. If this is a general phenomenon with anaerobic cultures, then clearly it undermines assessments of Y_{ATP} values as described previously (1, 50, 54), because less ATP then needs to be hydrolyzed to generate and maintain the membrane potential.

MODIFICATION OF RESPIRATORY CHAIN Phenotypic functional changes in the respiratory chains of microorganisms may occur in response to growth in certain nutrient-limited environments. For example, growth of the yeast *Candida utilis* in chemostat cultures that were either iron- or sulphate-limited effected a dramatic lowering of the nonheme iron and acid-labile sulphide contents of the cells' mitochondria with an accompanying loss of sensitivity to piericidin A and rotenone and loss of site-1 energy conservation (28, 29). Significantly, the yield value with respect to glycerol (the growth carbon substrate) was lowered concomitantly from 58.8 g mol^{-1} (average of 18 determinations) to 39.8 g mol^{-1} (average of 30 determinations).

Many bacterial respiratory chains probably contain more than one functional species of cytochrome oxidase, and terminal branching is therefore likely to be very common. In most cases this branching appears to involve electron transfer from a common, penultimate *b*- or *c*-type cytochrome to molecular oxygen via the available oxidases; however, in a number of bacterial species, branching seemingly is more complex and appears to involve *b*- and/or *c*-type cytochromes as well as multiple cytochrome oxidases (23). The major function of a branched respiratory system is undoubtedly to allow some flexibility in the exact route of electron transfer, thus enabling the cell to minimize the potential-

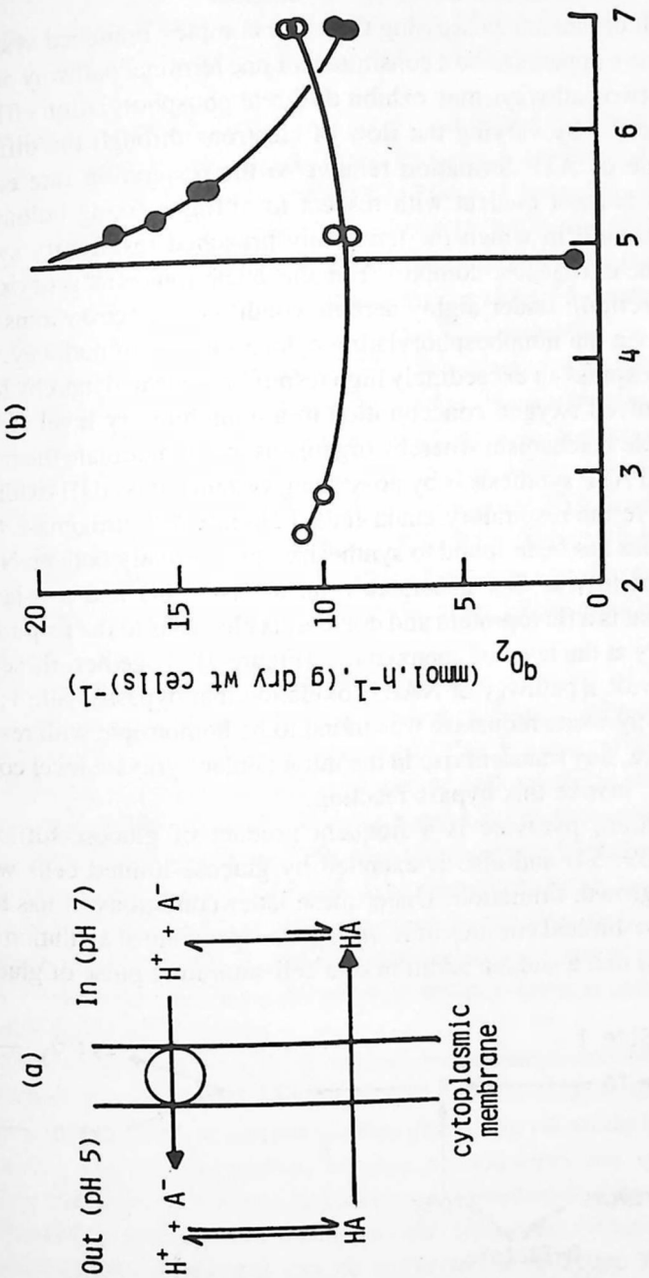


Figure 6 Influence of weak organic acids on the energetics of growth of microorganisms. The mechanism by which a transmembrane pH gradient (acid outside) might promote a cycling of such an acid over the plasma membranc is depicted in (*a*), while (*b*) shows the influence that the medium pH value was found to exert on the steady-state respiration rate of a chemostat culture of *Candida utilis* when growing in a mineral medium first acetate-limited (○) and then ammonia-limited with an excess of acetate (●); (see Ref. 20).

ly deleterious effects of certain growth environments (like the presence of cyanide) and to take maximum advantage of others.

However, with organisms possessing the more complex branched systems, where cytochrome *c* appears to be a constituent of one terminal pathway but not of the other, the two pathways may exhibit different phosphorylation efficiencies. In other words, by varying the flow of electrons through the different branches, the rate of ATP formation relative to the respiration rate can be modulated. This is most evident with respect to nitrogen-fixing cultures of *Azotobacter vinelandii* in which the terminally branched respiratory system helps to protect the nitrogenase complex from the deleterious effects of oxygen (respiratory protection); under highly aerobic conditions, electron transfer is probably routed via the nonphosphorylating cytochrome $b \rightarrow d$ pathway, thus allowing cells to express an exceedingly high respiration rate and thereby lower the ambient dissolved oxygen concentration to a noninhibitory level (23).

Another possible mechanism whereby organisms might modulate the rate of respiration-linked ATP synthesis is by possessing systems of NADH oxidation that do not involve the respiratory chain-linked NADH dehydrogenase (38). Thus, *K. aerogenes* has been found to synthesize constitutively both an NAD-linked pyruvate reductase that generates D-lactate (43, 44) and a D-lactate dehydrogenase that is a flavoprotein and donates its electrons to the respiratory chain, presumably at the level of coenzyme Q (Figure 7). Together, these two enzymes can provide a pathway of NADH oxidation that bypasses Site 1; and significantly, the pyruvate reductase was found to be homotropic with respect to pyruvate. Hence, any transient rise in the intracellular pyruvate level could, at least in theory, invoke this bypass reaction.

In this connection, pyruvate is a frequent product of glucose-sufficient aerobic growth (39, 54) and also is excreted by glucose-limited cells when relieved of their growth limitation. Under these latter conditions, it has been found with glucose-limited cultures of *K. aerogenes* (growing at a dilution rate of 0.2 h^{-1} or less) that a sudden addition of a cell-saturating pulse of glucose

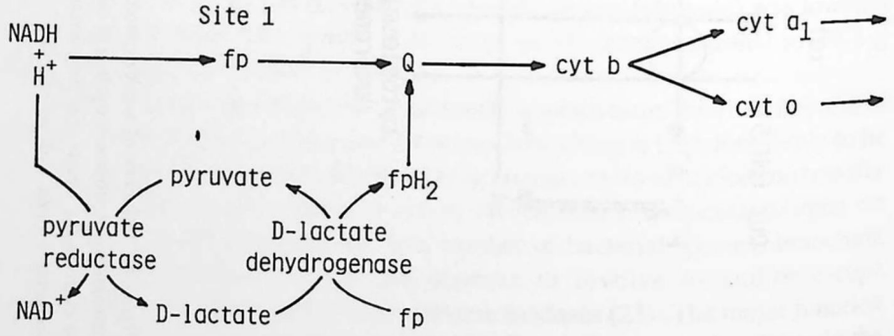


Figure 7 Possible mechanism of NADH oxidation that bypasses Site 1 of respiratory energy conservation.

provoked an immediate three- to four-fold increase in respiration rate, whereas the rate of cell synthesis increased only slowly. Hence, respiratory energy must have been dissipated at a high rate; and the bypass reaction shown in Figure 7 could make a significant contribution to this process.

METABOLIC UNCOUPLING It is well established (11) that, as with aerobic cultures, anaerobic cultures can consume carbon substrate at a high rate under conditions where biomass synthesis is severely impeded, if not totally prevented. Because with these anaerobic cultures, ADP phosphorylation is tightly coupled to substrate catabolism, mechanisms therefore must exist to hydrolyze the ATP so formed (27), or else they must be able to ferment carbon substrate via pathways that do not implicate net ATP formation.

Some modulation in the rate of ATP formation can be invoked by utilizing pathways of substrate catabolism that involve fewer substrate-level phosphorylation steps. Thus the fermentation of glucose to acetate and ethanol (plus formate) by *K. aerogenes* yields net 3 mol ATP per mol glucose, if the Embden-Meyerhof pathway is utilized, and 2 mol ATP per mol glucose if the Entner-Doudoroff pathway operates. Similarly, they can form but 1 mol ATP per mol glucose fermented if carbon substrate is converted to lactate via the latter pathway. But are they able to ferment glucose without any net ATP formation?

This question is relevant to the observed behavior of a glucose-limited anaerobic culture of *K. aerogenes* when suddenly relieved of the growth limitation (Figure 8). Here it was found that the rate of glucose catabolism immediately doubled, whereas the rate of cell synthesis increased only very slowly. Moreover, the rates of acetate and ethanol formation did not change, although there was an immediate onset of D-lactate formation with a concomitant formation of succinate and 2,3-butanediol (latter two not shown). So either these cells must possess some effective mechanism for turning over their ATP pool at a high rate, or else they must be able to ferment glucose without net ATP formation. The latter proposition initially seemed unlikely, but the clue clearly lay in the D-lactate.

As mentioned previously, *K. aerogenes* possesses constitutively a pyruvate reductase that generates D-lactate; but *E. coli* (its close relative) also is able to form D-lactate from dihydroxyacetone phosphate via methylglyoxal (3). Significantly, this methylglyoxal bypass circumvents the energy-conserving glyceraldehyde 3-phosphate dehydrogenase reaction and thus allows glucose to be fermented without ATP being formed. However, no more than 50% of the glucose carbon fermented can be converted to D-lactate via methylglyoxal, because 2 ATP equivalents are required to convert glucose to the two triose phosphates, and these must be recovered by subsequent glycolysis. Thus, only one of these triose phosphates can be converted to D-lactate via methylglyoxal, although a homolactic fermentation is possible if the other triose phosphate is

converted to lactate by way of the pyruvate reductase reaction. It is not fortuitous, we suggest, that a 1:1 stoichiometry was observed between the extra glucose fermented and the D-lactate formed subsequent to the glucose pulse (Figure 8) and the overall fermentation balance was close to that depicted in Figure 9.

Moreover, the enzymes necessary to effect this reaction now have been found to be present in *K. aerogenes* in amounts sufficient to effect D-lactate synthesis at the observed rate (56). Hence it is clear that the methylglyoxal pathway provides a perfect mechanism for *metabolically* uncoupling ATP formation from glucose fermentation; and we venture to suggest that this is precisely its functional significance.

Aerobically, of course, the methylglyoxal bypass (if present) can do no more than circumvent substrate-level phosphorylation reactions that, quantitatively, are not very significant. But if all the glucose carbon was converted to D-lactate via methylglyoxal, and the D-lactate was oxidized to pyruvate by D-lactate dehydrogenase, then glucose could be oxidized completely to pyruvate without invoking any net formation of ATP.

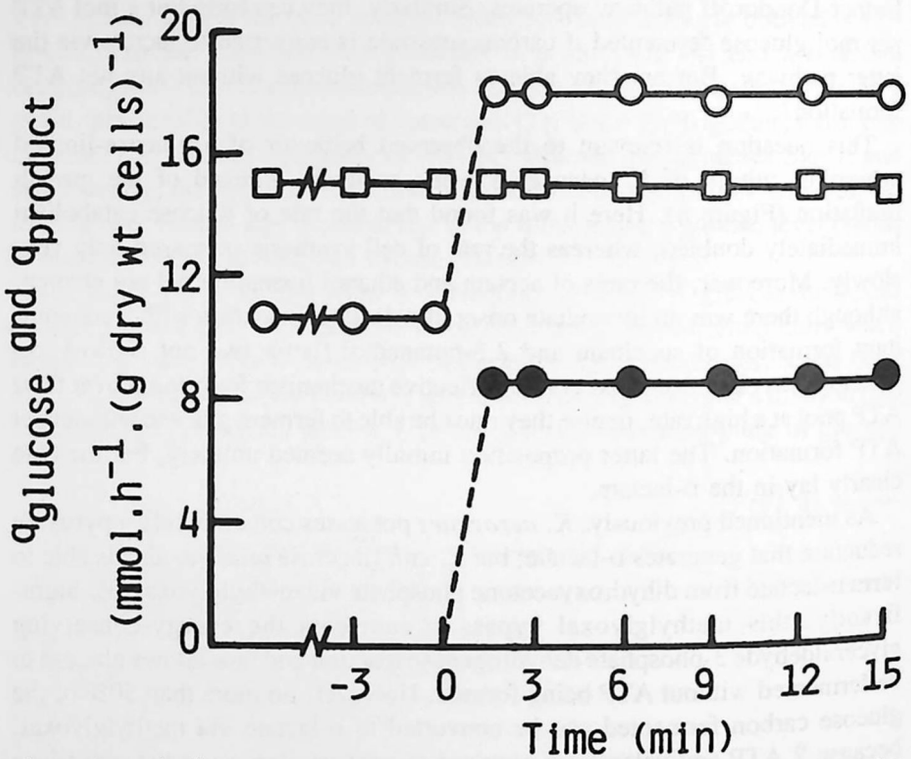
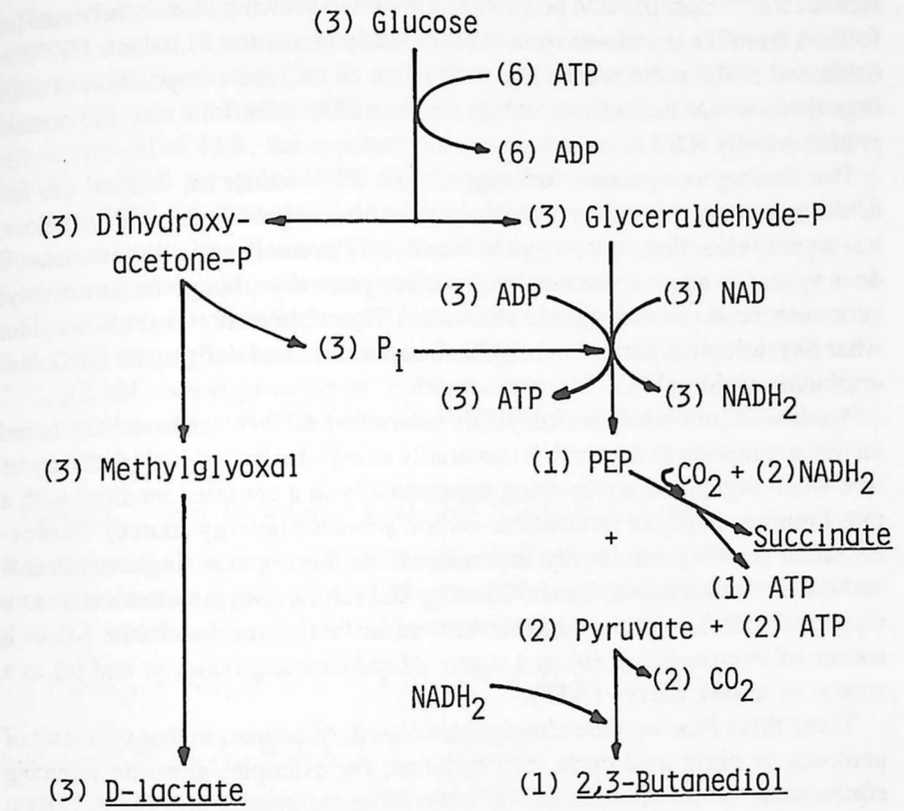


Figure 8 Changes in the specific rates of glucose consumption (○) and of products formation following the sudden addition of a cell-saturating pulse of glucose to a glucose-limited anaerobic culture of *Klebsiella aerogenes*. (□) Signifies specific rate of acetate plus ethanol formation, and (●) specific rate of D-lactate formation (see Ref. 56).



Overall reaction:

$$(3)\ C_6H_{12}O_6 \longrightarrow (3)\ C_3H_6O_3 + C_4H_{10}O_2 + C_4H_6O_4 + CO_2 + H_2O$$

Figure 9 Proposed pathway of glucose fermentation avoiding net formation of ATP. PEP = phospho*enol*pyruvate (see Ref. 56).

Interpretation of Yield Values

The multifarious nature of potential energy-spilling reactions present in microbial cells, as reviewed in the previous section, conspire to undermine confidence in assessments of that part of the energy flow that serves to fuel cell synthesis. Moreover, with regards to assessments derived from linear regression analyses of metabolic rate versus growth rate (7, 24, 54), there are yet other complications that stem from the fact that the macromolecular composition of organisms is variable; in particular, the cellular RNA content increases markedly with growth rate, and, in many cases, so too does cell size (17).

Hence, if Y^{max} specifies the properties of a culture growing at an infinite rate [as follows from the double-reciprocal relationship (Equation 3), where $1/Y^{max}$ is evaluated at the point where $1/\mu = 0$], then clearly the composition of such organisms would be far from reality: they would be of infinite size and contain predominantly RNA!

For these good reasons, we suggest that Y^{max} values so derived are not directly interpretable in physiological and/or bioenergetic terms. Nevertheless, it is equally clear that, whereas yield may be an extremely variable parameter, it does appear to attain some maximum value, generally (though not invariably) at or near the maximum-growth-rate value. Therefore it is relevant to consider what physiological parameters might circumscribe, and define, the maximum *attainable* yield value.

Analysis of microbial growth yields in terms of ATP fluxes have been based on the assumption that growth is essentially energy-limited, which well may be true when organisms are growing anaerobically in a complex medium with a rate-limiting supply of fermentable carbon substrate (energy source). However, when growing aerobically in a mineral medium with a single oxidizable carbon substrate that provides both energy and cell carbon, the situation is more equivocal. Here, carbon substrate serves, in fact, three functions: (*a*) as a source of intermediates; (*b*) as a source of reducing equivalents; and (*c*) as a source of usable energy (ATP).

These three functions are closely interrelated, of course, in that synthesis of pentoses or citric acid cycle intermediates, for example, generate reducing equivalents—and oxidation of the latter generate energy (ATP). A carbon substrate limitation therefore represents a complex situation in which, depending on the level of reduction of the carbon substrate and the redox state of other essential nutrients (e.g. ammonia or nitrate as nitrogen source), the culture may be basically either (*a*) carbon-limited (i.e. the nutrient carbon supply is limiting growth, and energy generation occurs in excess of the biosynthetic demands); or (*b*) energy-limited (including a limitation imposed by the cells' requirement for reducing equivalents). A carbon plus energy dual limitation is theoretically possible but, one might assume, uncommon. Under a true carbon limitation, the respiratory quotient would be relatively low, because the q_{CO_2} would be minimal and the excess reducing equivalents would be oxidized by respiration. The spillage of excess energy so generated would cause the Y_{O_2} value to be relatively low, but the $Y_{substrate}$ value would be maximal.

Conversely, with an energy-limited culture, a greater proportion of the carbon substrate would need to be catabolized to CO_2: thus, the q_{CO_2} would be relatively high and the $Y_{substrate}$ proportionately low. On the other hand, the Y_{O_2} value would be maximal, because energy spillage would be minimal. Although probably uncommon, a carbon-plus-energy dual limitation would be ideal for obtaining an optimal overall growth efficiency because, in this case, anabolism and catabolism would be perfectly matched.

These theoretical conclusions do, in fact, accord with experimental findings both with respect to growth of a single species on a number of different carbon substrates (61) and of different species on a wide variety of different compounds (30). In this latter study (30), which assumed a common bacterial carbon content of 48%, the apparent maximum attainable yield (1.43 g bacterial dry weight per g substrate carbon metabolized) represented a carbon conversion efficiency (cell carbon as a percentage of substrate carbon) of 68%. This was close to the value of 67% found with glucose-limited cultures of *K. aerogenes* growing at rates near to their maximum.

Assuming the elemental composition of *K. aerogenes* to be approximately $C_4H_7O_2N$, when growing at a near-maximal rate [actual values found (18) were $C_4H_{6.9}O_{1.7}N$, when growing at a dilution rate of 0.85 h^{-1}], and further assuming a carbon conversion efficiency of 67%, the following equation can be constructed:

$$C_6H_{12}O_6 + NH_3 + 2\ O_2 \rightarrow C_4H_7O_2N + 2\ CO_2 + 4\ H_2O.$$

This indicates a molar growth yield with respect to glucose of about 100, and with respect to oxygen (Y_{O_2}) of 50. A similar relationship for growth on glycerol, which again is close to that found experimentally (18), is:

$$2\ C_3H_8O_3 + NH_3 + 3\ O_2 \rightarrow C_4H_7O_2N + 2\ CO_2 + 6\ H_2O$$

($Y_{glycerol} = 50$; $Y_{O_2} = 33$).

Thus, accepting the evidence (23, 24, 52) that the respiratory chain of *K. aerogenes* contains but two sites of energy conservation (i.e. it lacks a *c*-type cytochrome), one can calculate the maximum ATP/O_2 gain when oxidizing, respectively, glucose and glycerol to completion and hence derive the respective minimum Y_{ATP} values from the above two growth equations. In this connection, glucose oxidation would generate 10 $NAD(P)H_2$, 2 fp_{red}, 2 ATP, and 2 GTP per mol, and would consume 6 mol oxygen. Thus, the ATP/O_2 maximum ratio would be $26/6 = 4.33$. Similarly, glycerol oxidation by this organism would generate 5 $NAD(P)H_2$, 2 fp_{red}, 1 ATP, and 1 GTP per mol, and would consume 3.5 mol oxygen. Here the ATP/O_2 maximum ratio would be $14/3.5 = 4.0$. Hence, the respective minimum Y_{ATP} values (uncorrected for maintenance) would be 11.7 for the glucose-limited culture and 8.4 for the glycerol-limited one.

This difference must, in part, be due to the extra bioenergetic demands of gluconeogenesis when glycerol is the carbon substrate; but even taking this into account, the corrected yield value (9.1) still is significantly lower than that for growth on glucose. The most probable explanation for this difference, we suggest, is that glycerol, being more reduced than glucose, imposes a carbon limitation on the culture as opposed to an energy limitation. But whatever the explanation, clearly growth on glycerol is energetically less efficient than

growth on glucose, and this example serves to illustrate the difficulties inherent in attempting to rationalize yield data in biosynthetic and bioenergetic terms.

Of relevance, in the above connection, are attempts that have been made to assess the energetic requirements for cell polymer synthesis from glucose and other carbon substrates on the basis of known biochemical pathways (9, 11, 51, 53, 66). Although these evaluations have been adjusted in recent years, as knowledge of the energy requirements of transport and of other metabolic processes has increased, the values so derived for the potential Y^{max}_{ATP} are uniformly much higher than those derived experimentally. For example (Table 1), when growing at a high dilution rate (0.85 h^{-1}) *K. aerogenes* was found to contain 69% protein, 19% RNA, 3% carbohydrate, and 6% lipid; to synthesize these polymers from glucose would require a minimum expenditure of energy (including transport processes) equivalent to 40.73 mmol ATP/g dry weight cells. This implies a potential Y^{max}_{ATP} value of 24.6 as compared with a value of 11.7 derived from the oxygen consumed in sustaining growth at this high rate.

Similarly, although the theoretical Y^{max}_{ATP} values for growth on other substrates (like lactate, acetate, methanol, and CO_2) are much lower than for growth on glucose (see 12, 52, 66), discrepancies of a comparable magnitude between these theoretical values and those derived experimentally are evident. In part, these may be attributed to the energetic requirements of maintenance functions; but even when these are taken into account, the experimentally determined Y^{max}_{ATP} values are not brought significantly closer to the theoretical maxima.

These large differences were first noted by Gunsalus & Shuster (11), who suggested, among other things, that energy might be dissipated by ATPase mechanisms; but no rationale was advanced as to why the latter should be manifest in optimally coupled energy-limited cultures. Later, on the basis of classical thermodynamics, Forrest (9) pointed out that the apparent low energetic efficiency of microbial growth correlated with a high outflow of entropy from the cells, and suggested that one of the major processes with which this was associated was cellular organization. In other words, whereas, when growing on substrates like glucose both the enthalpy and free energy of biosynthesis are small, there is a marked decrease in entropy that is made possible by a large outflow of entropy from the cells to their surroundings.

Table 1 ATP requirement for macromolecule synthesis from glucose, and the theoretical Y^{max}_{ATP} value for *Klebsiella aerogenes* with a cellular composition characteristic of rapidly growing cells

Component	ATP requirement (mmol per g)[a]	g Component per g dry cells	ATP equivalent
Protein	39.1	0.69	26.98
RNA	37.3	0.19	7.09
DNA	33.0	0.03	0.99
Polysaccharide	12.4	0.03	0.37
Lipid	1.5	0.06	0.09
(Transport)	—	—	5.21
		Total:	40.73

Theoretical Y^{max}_{ATP} = 1000/40.73 = 24.6

[a] Data of Stouthamer (52)

However, again, this rationalization provided no quantitative assessment; it did not explain why the experimentally determined Y^{max}_{ATP} values were uniformly 50% or less of the theoretical values, but simply added another "factor" (cellular organization) to the list of imponderables. Moreover, because most or all of the component reactions of cell synthesis are maintained far from their thermodynamic equilibrium values in growing cells, and many are irreversible, analyses in terms of classical (equilibrium) thermodynamics are not directly applicable. More relevant information might, however, be derived from the application of nonequilibrium thermodynamics [or, more particularly, mosaic nonequilibrium thermodynamics—so-called MNET (14, 70)].

Because, in an actively growing cell, catabolism is coupled to ATP generation, and the flow of ATP so created serves to drive biosynthesis, energetic losses might be incurred in both processes. For example, Stucki (55) has shown on the basis of linear nonequilibrium thermodynamics that, to maintain a net flow of ATP by oxidative phosphorylation, degrees of coupling less than 1 (i.e. $< 100\%$) are to be expected. By means of "conductance matching" he studied the optimization of four output functions of practical interest: net rate of oxidative phosphorylation; output power of oxidative phosphorylation; net rate of oxidative phosphorylation times efficiency; and output power times efficiency. He then evaluated the degree of coupling appropriate to each. From measurements of the phosphate potential extant in rat livers perfused at a "metabolic resting state," it was concluded that oxidative phosphorylation was optimized to the highest output power at lowest cost and with optimal efficiency where the degree of coupling was 0.97. With this degree of coupling, the thermodynamic efficiency (η_{opt}) would be 0.62 (55).

Reasoning along similar lines, Westerhoff and coworkers (14, 69, 70) analyzed the energetics of microbial growth in terms of phenomological and mosaic nonequilibrium thermodynamics. They determined the theoretical thermodynamic efficiency for each of six cases where the particular function was maximized (by varying the force ratio—i.e. the ratio of Gibbs free energy changes of anabolism, ΔG_a, and catabolism, ΔG_c) while the growth efficiency was kept optimal by adjusting the degree of coupling of the force ratio. The six conditions considered were (*a*) maximum rate of biomass synthesis ($-J_a$), (*b*) maximum power production ($-J_a \cdot \Delta G_a$), (*c*) maximum economic biomass synthesis ($-J_a \cdot \eta$), (*d*) maximum economic power production ($-J_a \cdot \Delta G_a \cdot \eta$), (*e*) maximum force ratio ($\Delta G_a/\Delta G_c$), and (*f*) maximum growth yield ($-J_a/J_c$, where J_c is the rate of catabolism).

From a comparison of the experimental thermodynamic efficiencies of growth of a number of microbial species (on different substrates) with the theoretical thermodynamic efficiencies appropriate to optimization of each of the above six conditions, they were able to conclude that growth on highly oxidized substrates corresponded to optimization for maximal rate of biomass synthesis at optimal thermodynamic efficiency, whereas growth on highly reduced substrates corresponded to optimization for maximal rate of biomass synthesis and/or maximal yield. Thus, this analysis provided not only a rationale for the widespread occurrence in microorganisms of mechanisms that can decouple catabolism from anabolism, and thereby serve to optimize the above functions, but indicated *why* actual yield values with respect to substrate catabolism and energy generation generally fell far short of those predicted on the basis of biochemical pathways.

A more detailed treatment of microbial growth energetics in terms of MNET is beyond the compass of this present review; the interested reader should consult recent publications on this and on related topics (12, 14, 67, 69, 70).

CONCLUSIONS

In this short review we have attempted to identify and analyze those processes that are fundamental to considerations of the physiological significance of yield values and to view these in the context of those theories that have been advanced to account for variations in this parameter. From this exercise, the broad conclusion can be drawn that yield values *per se* are not readily interpretable in precise bioenergetic and/or physiological terms, and, unless treated with considerable circumspection, they may lead to the formulation of concepts that are, at best, dubious. It is appropriate, therefore, to reiterate briefly those facts (or factors) that any interpretation of yield values should embrace. These are:

1. Heterotrophic microbial cells need to consume carbon substrate in order to grow, but the reverse is not true: cells do not have to grow to consume carbon substrate.
2. Similarly, growth demands an expenditure of energy (ATP hydrolysis), but cells do not have to grow to expend energy at a high rate; there is no obligatory coupling between ATP synthesis and growth.
3. Particularly when growing aerobically, carbon substrate-limited cultures are not necessarily energy-limited.
4. Organisms clearly can invoke energy-spilling reactions (both aerobically and anaerobically) that undermine assessments of the actual rate of ATP formation and thereby bedevil assessments of associated parameters such as P/O ratios and Y_{ATP} values (54, 65).
5. The maintenance energy rate and maximum yield value, derived from linear regression analysis of yield (or metabolic rate) versus growth rate, are essentially mathematical constants and not biological constants. In a strict physiological sense, both may vary with growth rate.
6. Carbon substrate sufficient cultures are not directly comparable with carbon substrate-limited cultures and allow of no comparisons to be made of the relative efficiencies with which energy (ATP) is being formed and/or consumed in biomass synthesis.

Clearly, the nub of the problem resides in the apparent capacity of microorganisms to partially or totally dissociate catabolism from anabolism, as circumstances require. Mosaic nonequilibrium thermodynamics offers a rational explanation for the widespread occurrence of this phenomenon, and physiological studies have revealed some (though probably by no means all) of the underlying mechanisms. Further physiological and genetical research is now needed to determine how these mechanisms can be purposefully manipulated (i.e. enhanced or eliminated), and whether the performance of organisms can thereby be steered to the optimization of industrially important processes as disparate as single-cell protein production (requiring maximum metabolic coupling) and waste-water treatment (requiring minimum metabolic coupling).

Clearly, in these areas, as well as in the field of primary and secondary metabolite overproduction (see 41), energy-spilling reactions play a crucial role. Hence it is in this aspect of microbial growth energetics that the interests of microbial physiologists, geneticists, bioenergeticists, and fermentation technologists become congruent and where one might expect to find in the coming years a substantial research investment. If this article helps to promote collaboration along these lines then we shall have achieved our objective.

Literature Cited

1. Bauchop, T., Elsden, S. R. 1960. *J. Gen. Microbiol.* 23:457–69
2. Belaich, J. P., Belaich, A., Simonpietri, P. 1972. *J. Gen. Microbiol.* 70:179–85
3. Cooper, R. A. 1975. *Biochem. Soc. Trans.* 3:837–40
4. Daldal, F., Fraenkel, D. G. 1983. *J. Bact.* 153:390–94
5. DeBoer, W. R., Kruyssen, F. J., Wouters, J. T. M. 1981. *J. Bact.* 145:50–60
6. DeMoss, R. D., Bard, R. C., Gunsalus, I. C. 1951. *J. Bact.* 62:499–510
7. Downs, A. J., Jones, C. W. 1975. *Arch. Microbiol.* 105:159–67
8. Epstein, W., Schultz, S. G. 1965. *J. Gen. Physiol.* 49:221–34
9. Forrest, W. W. 1969. *Symp. Soc. Gen. Microbiol.* 11:65–86
10. Gunsalus, I. C., Gibbs, M. 1952. *J. Biol. Chem.* 194:871–80
11. Gunsalus, I. C., Shuster, C. W. 1961. In *The Bacteria*, ed. I. C. Gunsalus, R. Y. Stanier, 2:1–58. New York: Academic. 572 pp.
12. Harder, W., van Dijken, J. P., Roels, J. A. 1981. In *Microbial Growth on C_1 Compounds*, ed. H. Dalton, pp. 258–69. London: Heyden. 377 pp.
13. Heath, E. C., Hurwitz, J., Horecker, B. L., Ginsberg, A. 1958. *J. Biol. Chem.* 131:1009–20
14. Hellingwerf, K. J., Lolkema, J. S., Otto, R., Neijssel, O. M., Stouthamer, A. H., et al. 1982. *FEMS Microbiol. Lett.* 15: 7–17
15. Hempfling, W. P., Rice, C. W. 1981. In *Continuous Culture of Cells*, ed. P. H. Calcott, 2:99–125. Boca Raton: CRC Press. 209 pp.
16. Herbert, D. 1958. *Symp. 7th Intern. Congr. Microbiol.* 381–96
17. Herbert, D. 1961. *Symp. Soc. Gen. Microbiol.* 11:391–416
18. Herbert, D. 1976. In *Continuous Culture*

6: Applications and New Fields, ed. A. C. R. Dean, D. C. Ellwood, C. G. T. Evans, J. Melling, pp. 1–30. Chichester: Ellis Horwood. 364 pp.
19. Hueting, S., DeLange, T., Tempest, D. W. 1979. *Arch. Microbiol.* 123:183–88
20. Hueting, S., Tempest, D. W. 1977. *Arch. Microbiol.* 115:73–78
21. Hurwitz, J. 1958. *Biochim. Biophys. Acta* 28:599–609
22. James, A. M., Djavan, A. 1980. *Microbios* 29:171–83
23. Jones, C. W. 1977. *Symp. Soc. Gen. Microbiol.* 27:23–59
24. Jones, C. W., Brice, J. M., Edwards, C. 1977. *Arch. Microbiol.* 115:85–93
25. Katz, J., Rognstad, R. 1978. *Trends Biochem. Sci.* 3:171–74
26. Ko, R. Y. C., Edwards, V. H. 1975. *Biotechnol. Bioengin.* 17:965–83
27. Lazdunski, A., Belaich, J. P. 1972. *J. Gen. Microbiol.* 70:187–97
28. Light, P. A. 1972. *J. Appl. Chem. Biotechnol.* 22:509–26
29. Light, P. A., Garland, P. B. 1971. *Biochem. J.* 124:123–34
30. Linton, J. D., Stephenson, R. J. 1978. *FEMS Microbiol. Lett.* 3:95–98
31. Mallette, M. F. 1963. *Ann. N.Y. Acad. Sci.* 102:521–35
32. Marr, A. G., Nilson, E. H., Clark, D. J. 1963. *Ann. N.Y. Acad. Sci.* 102:536–48
33. Michels, P. A. M., Michels, J. P. J., Boonstra, J., Konings, W. N. 1979. *FEMS Microbiol. Lett.* 5:357–64
34. Monod, J. 1942. *Récherches sur la Croissance des Cultures Bactériennes*. Paris: Hermann. 210 pp.
35. Monod, J. 1950. *Ann. Inst. Pasteur* 79:390–410
36. Neijssel, O. M. 1976. *The significance of overflow metabolism in the physiology and growth of Klebsiella aerogenes*. PhD thesis, Univ. Amsterdam. 52 pp.
37. Neijssel, O. M. 1977. *FEMS Microbiol. Lett.* 1:47–50
38. Neijssel, O. M., Sutherland-Miller, T. O., Tempest, D. W. 1978. *Proc. Soc. Gen. Microbiol.* 5:49
39. Neijssel, O. M., Tempest, D. W. 1976. *Arch. Microbiol.* 107:215–21
40. Neijssel, O. M., Tempest, D. W. 1976. *Arch. Microbiol.* 110:305–11
41. Neijssel, O. M., Tempest, D. W. 1979. *Symp. Soc. Gen. Microbiol.* 29:53–82
42. Novick, A., Szilard, L. 1950. *Science* 112:715–21
43. Pascal, M.-C., Pichinoty, F. 1965. *Biochim. Biophys. Acta* 105:54–69
44. Pascal, M.-C., Pichinoty, F. 1966. *Bull. Soc. Chim. Biol.* 48:583–93
45. Pine, M. J. 1972. *Ann. Rev. Microbiol.* 26:103–26
46. Pirt, S. J. 1965. *Proc. Roy. Soc. London, Ser. B* 163:224–31
47. Pirt, S. J. 1982. *Arch. Microbiol.* 133:300–02
48. Pöhland, D., Ringpfeil, M., Behrends, U. 1966. *Z. Allg. Mikrobiol.* 6:386–95
49. Powell, E. O. 1967. In *Microbial Physiology and Continuous Culture*, ed. E. O. Powell, C. G. T. Evans, R. E. Strange, D. W. Tempest, pp. 34–56. London: H. M. S. O. 261 pp.
50. Rosenberger, R. F., Elsden, S. R. 1960. *J. Gen. Microbiol.* 22:726–39
51. Stouthamer, A. H. 1973. *Antonie van Leeuwenhoek*, 39:545–65
52. Stouthamer, A. H. 1977. *Symp. Soc. Gen. Microbiol.* 27:285–315
53. Stouthamer, A. H., Bettenhaussen, C. W. 1973. *Biochim. Biophys. Acta* 301: 53–70
54. Stouthamer, A. H., Bettenhaussen, C. W. 1975. *Arch. Microbiol.* 102:187–92
55. Stucki, J. W. 1980. *Eur. J. Biochem.* 109:269–83
56. Teixeira de Mattos, M. J., Tempest, D. W. 1983. *Arch. Microbiol.* 134:80–85
57. Tempest, D. W. 1969. *Symp. Soc. Gen. Microbiol.* 19:87–111
58. Tempest, D. W. 1978. *Trends Biochem. Sci.* 3:180–84
59. Tempest, D. W., Meers, J. L. 1968. *J. Gen. Microbiol.* 54:319–25
60. Tempest, D. W., Meers, J. L., Brown, C. M. 1970. *J. Gen. Microbiol.* 64:171–85
61. Tempest, D. W., Neijssel, O. M. 1980. In *Diversity of Bacterial Respiratory Systems*, ed. C. J. Knowles, 1:1–31. Boca Raton: CRC Press. 263 pp.
62. Ten Brink, B., Konings, W. N. 1982. *J. Bact.* 152:682–86
63. Torriani, A. 1960. *Biochim. Biophys. Acta* 38:460–70
64. Tsang, M. L.-S., Schiff, J. 1976. *J. Bact.* 125:923–33
65. Van der Beek, E. G., Stouthamer, A. H. 1973. *Arch. Mikrobiol.* 89:327–39
66. Van Dijken, J. P., Harder, W. 1975. *Biotechnol. Bioengin.* 17:15–30
67. Walz, D. 1979. *Biochim. Biophys. Acta* 505:279–353
68. Watson, T. G. 1970. *J. Gen. Microbiol.* 64:91–99
69. Westerhoff, H. V. 1983. *Mosaic non-equilibrium thermodynamics and the control of biological free energy transduction*. PhD thesis. Univ. Amsterdam. 367 pp.
70. Westerhoff, H. V., Lolkema, J. S., Otto, R., Hellingwerf, K. J. 1982. *Biochim. Biophys. Acta* 683:181–220
71. Wouters, J. T. M., Buijsman, P. J. 1980. *FEMS Microbiol. Lett.* 7:91–95

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DEEP-SEA MICROBIOLOGY

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INTRODUCTION

The *Challenger* Expedition (1873–1876) is commonly credited as the historical beginning of deep-sea biology. The finding of live specimens at great depths obliterated the azoic-zone theory that had been suggested in the 1840s by Edward Forbes, a leading British oceanographer. Based on observations of the diminishing quantities of bottom fauna with increasing depth, it had been believed that no life existed below approximately 600 m. The death of this theory was accompanied by the dispatch of *Bathybius*. Because this foremost and universal microorganism had promised, however briefly, a splendid beginning of deep-sea microbiology, the affair deserves to be briefly reported.

During the 1850s Thomas Henry Huxley, not content with the azoic-zone concept, sought opportunities to examine deep-sea sediment samples. In 1857

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the steamer HMS *Cyclops* was conducting a series of soundings in the North Atlantic in preparation for the laying of a cable. On Huxley's request, the ship's medical officer preserved in alcohol sediment samples that had been entrapped on equipment brought up from the bottom. In this material Huxley (45) discovered the coccolithophores, which he proposed to be "produced by independent organisms." Under the strong influence of early Darwinism and of Haeckel's protoplasm theory, he postulated that the whitish, mucoid material encasing the coccolithophores might represent Haeckel's *Urschleim* (primordial slime), which he named *Bathybius haeckeli*. From follow-up samplings taken during the *Challenger* Expeditions it became clear that Huxley's observed *Urschleim* was in actuality an alcohol precipitate of organic debris. He admitted his error gallantly.

This affair, which was aptly retold and documented by Rehbock (115), occurred at a time when nonmedical microbiology was in its infancy. Although the ubiquity and diversity of microorganisms began to gain acceptance during the 1860s, the conceptual and methodological basis for studying microbial life in the sea, other than by use of the microscope, was not yet available to the scientists of the *Challenger* Expedition. This fact, and the general inaccessibility of the study area, may have contributed to the relatively late and sporadic start of deep-sea microbiology.

Better equipped by Pasteur's new concepts, Certes (18), during the *Travaillier* and *Talisman* Expeditions (1882–1883), examined sediment and water collected from depths to 5000 m and found bacteria in almost every sample. He noted that bacteria survive great pressures and may live in a state of suspended animation (19). During a crossing of the Atlantic by commercial liner in 1886, Fischer (36) plated water samples on solidified bacteriological media, a technique that had just been introduced by Koch (74). He found "very few" colonies per ml in waters below 1100 m (37). In 1904 Portier used a sealed and autoclaved glass tube as a bacteriological sampling device [described by Richard (117)] and reported counts of colonies from various depths and locations.

Two monographs on marine microbiology, Benecke (8) and ZoBell (175), discuss these beginnings in detail. It is apparent from both of these sources, however, that a definable deep-sea microbiology did not exist before the middle of this century, with the exception of the efforts of Certes and Portier. In 1949, ZoBell & Johnson (179) started work on the effect of hydrostatic pressure on microbial activities. ZoBell's participation in the Danish *Galathea* Expedition (1950–1952) and his subsequent work resulted in several publications that were later discussed comprehensively in a number of reviews (98, 102, 176, 177).

The microbiological aspects of barobiology have been represented in symposia volumes edited by Zimmermann (174), Sleigh & Macdonald (134), and Brauer (10), emphasizing hydrostatic pressure as an environmental or experimental physicochemical variable. Treatises on pressure as an ecological, physiological, and molecular parameter in microbial metabolism were written by Landau (79), Kinne (73), Morita (98, 100), Marquis (93), Marquis & Matsumura (95), and Landau & Pope (82). Two recent articles, by Marquis (94) and by Jannasch & Wirsen (63), were prepared for a general biological and an oceanographic audience, respectively. The format of the latter publication was used in part for the preparation of the present chapter, which, as a whole, focuses on material not covered by earlier reviews.

CONCEPTS AND DEFINITIONS

The definition of "deep" is open to intelligent choices. Light and photosynthetic production occur in the upper 300-m layer of the oceans. Temperature decreases with depth until an almost constant 3°C (± 1°) is reached below the thermocline (30–100 m). To encompass a zone typified for its remoteness from the productive surface waters and attendant seasonal and topographically derived fluctuations in physical properties, the deep sea is conveniently defined as waters of 1000 m and below. It underlies 88% of the global area covered by seawater and includes 75% of the oceans' total volume. The average depth of the oceans, derived by dividing total volume by total area, is 3800 m. The maximum depth in the trenches can reach 11,000 m, but the volume of seawater below the "abyssal plain" (about 6000 m) is only 0.1% of the total.

Considering the fact that seawater supports life at all depths, and conveniently assuming a 38-m thickness of the continental biosphere, it follows that 99% of the globe's entire biosphere consists of seawater. Of this amount, 62% exists at 2°C and hydrostatic pressures of 100 atm and higher. This fact is of ecological importance when regarding the thin photosynthetically productive skin of the globe as compared to the enormous volume of the deep sea as the universal and ultimate sink.

Theoretical settling rates of phytoplankton, as well as the flocculant aggregates of particulate matter consituting "marine snow" (131, 132), range from 1.0 to 0.1 m day^{-1}, or 5000 m in 1–50 years (157). It is generally estimated that about 95% of the organic matter produced photosynthetically in the surface waters is recycled in the upper 100–300 m (12, 42, 43), the availability of light and nutrients being geographically highly variable. According to Honjo et al (44), the larger portion of the remaining organic matter is decomposed in the mesopelagic zone (about 300–1200 m) with very little penetration into the bathypelagic zone (from 1200 m to the bottom). Only about 1% of photosynthetically produced organic carbon reaches the deep-sea floor. Honjo states that "the decomposition rate of organic carbon in the deep water can be estimated to

be about 2.2 mg C/m^2/day, a value consistent with rates of oxygen consumption estimated by other investigators."

This general figure was also presented in a recent discussion by Jørgensen (69), [based on data compiled by Suess (142)], stating that "the fraction [of local phytoplankton production] which reaches the sediment is mainly a function of water depth" and "decreases gradually to reach about 1% in pelagic sediments at 5000–6000 m depth." Thus, the major nutritional characteristics of the deep sea as a habitat for organisms are the relatively low input of organic carbon and its consumption during vertical transport over a vast space. As a corollary, adaptations to oligotrophy (see below) are common.

The above data are derived from studies conducted with sediment traps that may underestimate the flux of large and sparse particles. From many observations it appears that the typical benthic and epibenthic macrofauna (the latter, including bottom-feeding fishes, living *on* rather than *in* the sediments) are scavengers feeding on larger chunks of food such as the carcasses of fishes, decapods, and large shrimp (40). These materials sink to the deep-sea floor at rates that preclude a substantial en route decomposition (500–50 m hr^{-1}, or 5000 m in 10–100 hr) (157). Fecal pellets with sinking rates of 200–50 m day^{-1} (for zooplankton) and up to 2700 m day^{-1} (for salps) constitute another considerable input of particulate organic carbon to the deep sea (15, 75, 92, 154). It is not clear at this time whether marine snow, animal carcasses, or fecal pellets represent the major input of organic matter from surface waters to the deep sea.

With the few exceptions of enclosed basins, such as the Black Sea, the Cariaco Trench off Venezuela, and various Norwegian and Canadian fjords, deep-sea water as well as the upper layers of most deep-sea sediments are oxygenated. The concentrations of dissolved oxygen rarely fall below one half of air saturation levels (about 4 mg liter^{-1} or 130 μM), a fact largely due to the extremely low rates of oxygen consumption observed in this oligotrophic environment. The term "oligotrophy," originally coined for habitats of low-nutrient content, has been recently used to designate growth characteristics of organisms capable of coping with low-nutrient conditions (108, 130).

The particular temperature regime of 3°C ± 1° for most of the deep sea is apt to select for low temperature–adapted "psychrophilic" microorganisms (7, 46, 47, 125), defined by growth optima between 8–15°C and upper limits near 25°C. Their predominant occurrence in the deep sea has been generally established (101, 164). Hydrostatic pressure increases by approximately 1 atm (= 1.033 kg cm^{-2}, = 1.013 bar, = 1.013 × 10^2 kPa) for every 10 m of depth. The deviation from this relationship amounts to about +9.4 atm at 5000 m and to about +29 atm at 10,000 m depth and 30° latitude (124), mainly due to the compressibility of seawater and changes in gravitation (the earth's radius increases with decreasing latitude).

Growth or metabolic activity of natural populations or pure cultures of bacteria are "barotolerant" to various degrees. In other words, they tolerate increased hydrostatic pressure, as commonly measured by growth on specific media, up to different maximum pressures. The term "barophilic," first used by ZoBell & Johnson (179), is defined today as optimal growth at pressures higher than 1 atm (normal atmospheric pressure) or by a requirement of increased pressure for growth, i.e. no growth at normal atmospheric pressure (see Figure 2). As discussed below, both pressure- and temperature-adapted bacteria may lose their specific growth characteristics under changing environmental conditions.

IN SITU STUDIES

The problems involved in the retrieval of samples from great depths, notably the critical changes of temperature and pressure, can best be prevented by conducting experiments in situ, i.e. directly on the deep-sea floor. Such in situ incubation experiments have been performed at the expense of desirable sophisticated procedures—such as repeated subsampling—that permit the calculation of activity rates. Yet simple end-point measurements of the degradation of certain solid materials were of practical use, especially in the case of studies on manila, hemp, and wood (104), and have supported the general notion of a suppression of microbial activity at great depths.

Quantitative studies with liquid media (^{14}C-labeled acetate, glucose, mannitol, and casamino acids) inoculated in situ were preceded by experiments with mixed and pure cultures submerged and incubated in the deep sea (55). Parallel samples were kept as controls for incubation at 1 atm and deep-sea temperature. These studies were based on the assumption that most surface-originated, sedimenting materials carry microorganisms down with them and that the activity of these surface-derived microorganisms might be affected not only by the decreasing temperature but also by the increasing pressure. Indeed, the data indicated markedly reduced rates of microbial activities by the increase of pressure alone. Deep-sea incubation studies with a controlled in situ inoculation of sterile media became possible through the use, to depts of 4000 m, of the research submersible *Alvin* (57). Inoculated samples were deposited on the sea floor for up to one year of incubation. Parallel samples were returned for control incubations at 1 atm in the laboratory. This approach has been used for studying the decomposition of seaweeds, wood, paper, chitin, agar, and starch, as well as the above-mentioned radiolabeled dissolved organic substrates (57, 165).

For more extensive and quantitative studies on dissolved substrates, a "syringe array" was designed. Six 200-ml syringes containing radiolabeled substrate were filled in situ through a joint inlet nozzle by rotary movement of

Alvin's mechanical hand; they were incubated in situ with a control set for incubation in the laboratory (53). This approach has been used extensively at depths to 4000 m and at different geographic locations, including the Galapagos Rift area, for measurements of in situ CO_2 fixation (see below). Advantages over previous methods of in situ inoculation (57) are that no pressure differential exists during filling and that pressure housings are unnecessary.

The data of these experiments with natural microbial populations demonstrated invariably that: (*a*) the conversion of organic substrates was slower in situ than in the 1-atm controls incubated at the same temperature; (*b*) in situ conversion rates were similar when the inoculum was taken near the surface or in deep water; and (*c*) the effect of increased pressure on metabolic rates was substrate-dependent. These general results were confirmed when pressure-retaining samplers were used over a wider depth range (see below).

Experimentation with *Alvin* on the deep-sea floor is restricted by weather, expense, and the present depth limitation of 4000 m. Therefore, the possibilities of using "free vehicles" for microbiological experiments have been explored, and the vehicles have been found to be extremely useful. Free vehicles—also called "bottom landers" or "tripods"—are untethered instrument packages that descend to the deep-sea floor for various automated functions and measurements and return to the surface after the timed release of an anchor weight. Since Isaacs's (49) original work, tripods of various configurations and degrees of technical sophistication have been used (54, 60, 120, 162). Tripods can be used to practically any depth. Most of them are equipped with acoustic tracking devices and can be recalled on command by acoustic release of the anchor weight.

For studies in deep-sea microbiology, timer-operated tripods have been used for measuring: (*a*) microbial growth in a peptone–yeast extract medium (129), (*b*) microbial nitrate metabolism (158), (*c*) transformation of radiolabeled organic substrates injected into various levels of the upper sediment layers (60), (*d*) decomposition of substrates contained in compartmentalized tubes and inserted into the sediment (165), and (*e*) trapping and incubation of deep-sea amphipods in the presence of a radiolabeled solid food source for comparative studies on the metabolism of deep-sea scavengers and their intestinal microflora (54, 166).

Due to sedimentation, microbial activity in the very top layer of deep-sea sediments can be expected to be orders of magnitude higher than in the water column directly above, as well as in the sediment layers only centimeters below the surface. It was found, for example, that the respiration of acetate and its incorporation into particulate organic carbon decreased within the upper 7 cm of deep-sea sediment. This trend was evident for both in situ incubated samples and their 1-atm controls, although the overall rate of substrate conversion was substantially higher in the latter (60). In another study, deep-sea amphipods

were captured and incubated in situ with a ^{14}C-labeled protein food source (54); the conversion of carbon within various biochemical fractions in the amphipod tissue differed from that in the gut tract and intestinal microflora. This approach offers the means for studying metabolic strategies of procaryotes and eucaryotes under deep-sea conditions. The first results suggest lipid energy storage and enhanced microbial growth within the amphipod guts as compared to growth on the uningested food material (166).

GROWTH STUDIES ON DEEP-SEA BACTERIA

Natural Populations

All in situ inoculation-incubation experiments have the distinct disadvantage of being limited to end-point determinations. For time-course experiments on growth and enrichments of deep-sea bacteria, samples had to be retrieved, repressurized, and incubated at both in situ pressure and temperature. This approach resulted in the finding of barophilic and psychrophilic bacteria reviewed in detail by ZoBell (176) and Morita (98, 99, 101). Recent work focused on the temperature and pressure sensitivity of these organisms and the necessary techniques in avoiding changes of deep-sea conditions during sampling and laboratory procedures. Two such samplers were designed and constructed with working depths of 2000 m and 6000 m, respectively (64, 66). They are lowered on a cable and triggered by a messenger to obtain a 1-liter sample without subjection to damaging shear forces. There is sufficient insulation in the housing to prevent temperature damage to psychrophilic organisms on retrieval through the thermocline. Transfer units permit introduction and removal of 13-ml subsamples without affecting the pressure within the sample. Thus the samplers may also be employed as culture chambers.

In order to increase the efficiency of sampling at sea, a filter sampler was subsequently developed for the in situ 200-fold concentration of 3-liter water samples (58). This instrument can be sterilized and reused at sea and as many individual undecompressed samples can now be collected on a cruise as the number of transfer-storage units available. In the laboratory the samples may be transferred into the above-mentioned culture chambers that are, for this purpose, filled with seawater medium and prepressurized.

Use of these instruments for incubation of samples from various depths revealed that upon decompression to 1 atm, substrate metabolism invariably increased (62, 64). The extent of this effect appears to depend on the type of substrate used. For example, the rate of acetate metabolism was much less affected by pressure than that of glutamate (Figure 1). Two interpretations are possible: (*a*) substrates enrich specifically for types of microorganisms, differing by their degree of barotolerance; or (*b*) substrate-specific transport or metabolic pathways are influenced differently by pressure, irrespective of the organism carrying out the conversion.

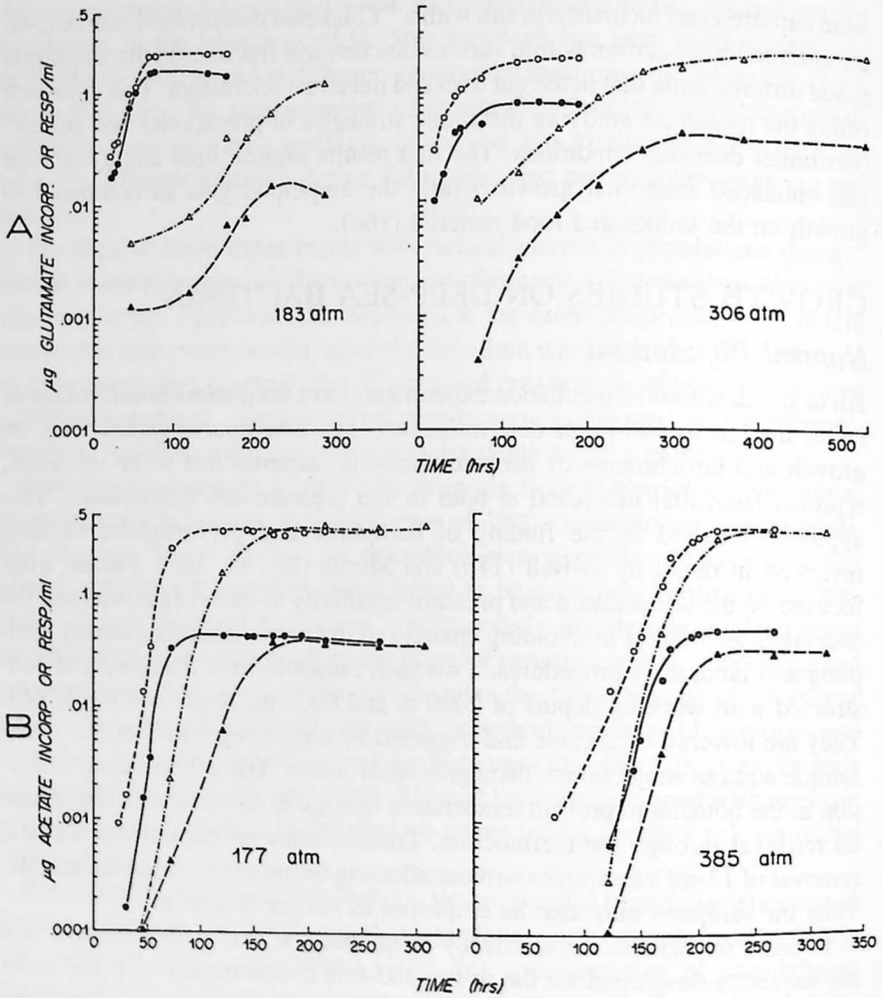


Figure 1 Incorporation *(filled symbols)* and respiration *(open symbols)* of ^{14}C-labeled sodium glutamate (*A*) and sodium acetate (*B*) added to seawarer (initial concentration 0.5 μg ml^{-1}) collected from two depths and incubated (3.0 ± 0.5°C) at in situ pressure without prior decompression *(triangles)* and at 1 atm *(circles)*. *(From 62)*

Different technical principles for the procurement and subsampling of undecompressed deep-sea microbial populations were used in instrumentation developed by Colwell & Tabor (20, 144, 145). Results of studies with this apparatus, conducted at several oceanic locations and using various substrates at low concentrations, generally supported the trends discussed above, i.e. stimulation of substrate utilization upon decompression. When glutamate was introduced into pressurized samples at levels of approximately 50 μg liter^{-1} (84, 145), microbial activity ceased before 2% of the substrate was utilized. This response to pressure was more pronounced than the 30% substrate utilization observed in studies where glutamate was introduced at levels of 500–6000 μg liter^{-1} (62). Although mechanisms for these responses are presently not clear, it appears that their magnitude is dependent upon the substrate employed and not the result of secondary effects such as limitation by inorganic nutrients or oxygen or alterations in pH.

Pure Culture Isolates

Work on deep-sea bacteria isolated at 1 atm and studied at various temperatures and pressures has been reviewed extensively by Morita (98) and by Marquis & Matsumura (95). Since then a major advance has been the isolation and unequivocal demonstration of the existence of barophilic bacteria (170, 171). These organisms were obtained from pressurized enrichments of decomposing deep-sea amphipods by growth in serial dilutions made in an organic-rich silica-gel medium (28). The samples were decompressed only for the short periods necessary for manipulation and transfer procedures. The first isolate to be described in these studies (170), a spirillum-like organism obtained from 5800-m depth, possessed a maximum growth pressure of 1000 atm and a broad growth optimum around 500 atm, with a minimum generation time of 8–9 hr at 2–4°C. Growth at 1 atm was at least 30-fold lower than the optimum, clearly reflecting the barophilic character of the organism.

An obligate barophilic organism was obtained (171) from an amphipod collected in the Mariana Trench (10,500-m depth). With a generation time of 33 hr, this strain grew relatively well at 1000 atm and 2°C as compared to its optimal growth (generation time of 25 hr) at 690 atm. At less than 350 atm the organism failed to grow, indicating its requirement for increased pressure. Barophilic bacteria were also described by Deming et al (25–27) in studies on members of the bacterial populations isolated from the intestinal tracts of deep-sea amphipods and sediment-dwelling holothurians.

The types of microbial growth responses to pressure are presented schematically (Figure 2) because the available data have not been obtained under comparable conditions. The relationships between the four curves are arbitrary in a quantitative sense. They do reflect an overall linear or nonlinear decrease of activity with increasing pressure or depth (*D* in Figure 2). From results obtained with natural microbial populations it appears likely that the various growth responses to pressure are strongly affected by the types of substrates used, i.e. the particular metabolic pathways and enzyme systems involved.

The notion that the abundance of psychrophilic bacteria in the deep sea may be generally linked to a high barotolerance (172) was only partly confirmed (55, 164). Psychrophilic bacteria have been described to be widespread on land, in fresh water, and in surface seawater (46, 47, 101, 125). Similarly, high bacterial barotolerance can be found in mesophilic isolates from marine surface waters or even garden soil (76, pp. 91–103). It appears, however, that most

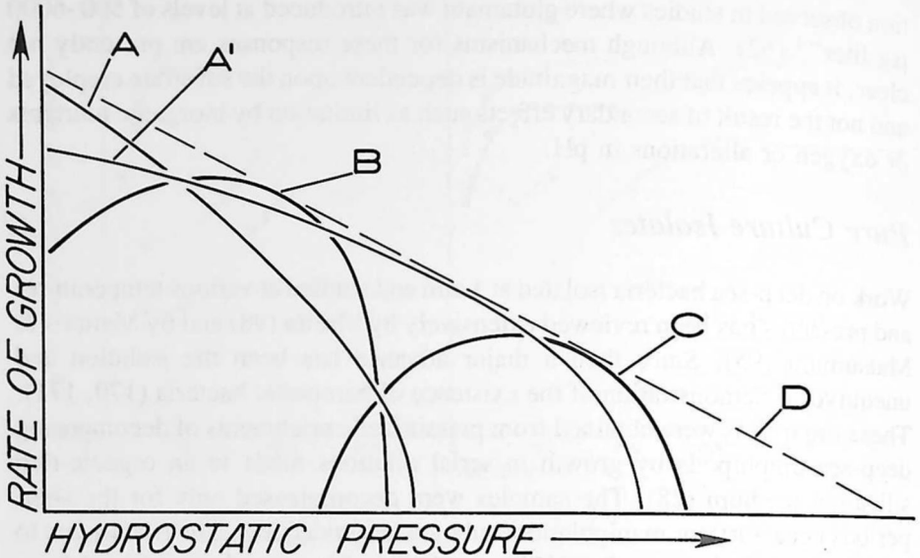


Figure 2 Scheme of microbial growth responses to hydrostatic pressure, based on available data: *A* and *A'*, barotolerance varying by degree and range; *B*, barophile; *C*, obligate barophile; *D*, overall decrease of activity. (From 53)

barophilic deep-sea isolates are also psychrophilic (168, 172). Whether any obligate interdependence between these growth characteristics exists is unclear at this point.

Although the thermal inactivation and death of psychrophilic bacteria at moderate temperatures are well known and carefully considered during sampling and isolation procedures (168, 172), analogous studies on the viability of decompression-sensitive bacteria cannot be conducted unless pure cultures isolated in the absence of decompression can be obtained. On the other hand, general experience has shown that barophilic bacteria can readily be isolated in spite of short periods of decompression. Indeed, such procedures have been successful in obtaining an obligate barophile (171), even though it is moderately sensitive to decompression (90% killed in 5 hr) (169). However, these results do not preclude the existence of highly decompression-sensitive microorganisms that might not survive even very short periods of decompression. The technology for completely eliminating decompression during the isolation of deep-sea microorganisms is now available (65).

The above-mentioned filter sampler can be used for the transfer of undecompressed 13-ml concentrates (1:200) of deep-water samples (from depths to 10,000 m) into an isolation-purification chamber (Figure 3). Although helium at high pressure is not physiologically neutral (see 11), data indicate that growth of barotolerant bacteria and other organisms under a compressed atmosphere of this gas is better than in hydrostatic pressure controls (97, 151, 152), even at pressures up to 500 atm (147). Studies have also shown that

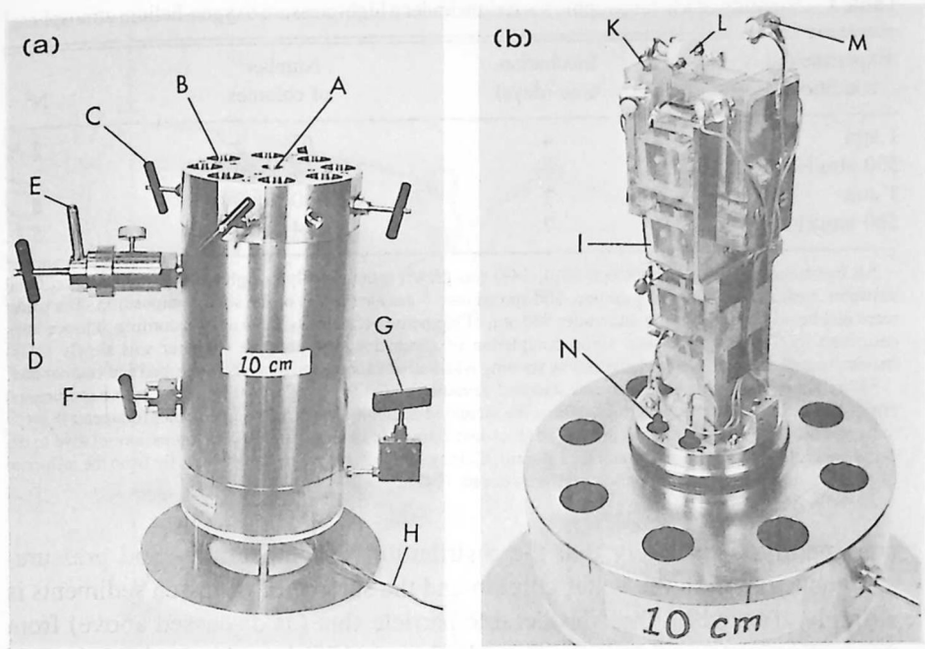


Figure 3 Chamber for the isolation of microorganisms at pressures up to 1000 atm in the absence of decompression.

(*a*) Isolation chamber assembled. *A*, viewing window; *B*, reset end cap bolts; *C*, valves for sample transfer into four vials; *D*, handle for horizontal movement of streaking loop; *E*, lever for rotary movement of streaking loop; *F*, handle for movement of agar plates; *G*, valve for pressurization; and *H*, wires for illumination and loop sterilization. (*b*) Bottom end cap with inner parts of isolation chamber (top removed); *I*, nine agar plates; *K*, four vials for liquid medium; *L*, ceramic loop holder with streaking loop; *M*, five halogen lamps; and *N*, wire penetrators. (From 65)

precise control of oxygen fugacity in hyperbaric chambers is routinely possible (146, 148). Therefore, the traditional agar plating technique may be used for the isolation of bacteria in a hyperbaric chamber with an oxygen-helium gas phase (Table 1). The chamber (Figure 3) can be prepressurized to 1000 atm and facilitates streaking of cell suspensions on small petri dishes under a window with an internally resterilizable loop. Colonies can be transferred into 8-ml vials containing liquid media, and cell suspensions can be withdrawn from the isolation chamber and transferred into prepressurized 1-liter culture chambers.

Figure 4 represents data on three organisms grown in the same medium but isolated under different conditions. The barotolerant (strain 40K) and the barophilic (strain 51A-1) organisms were obtained from decompressed samples, whereas the other barophilic organism (strain 72) was isolated in the absence of decompression.

Observations by Yayanos et al (172), that the pressure optima for growth of barophilic isolates is almost always identical with the in situ pressure of the sample origin, also were made by us in a few cases (Figure 4), but not

Table 1 Viability of a heterotrophic bacterium under a high-pressure oxygen-helium atmosphere[1]

Experimental conditions	Incubation time (days)	Number[2] of colonies	N[2]
1 atm	4	65 ± 5	2
500 atm/He	4	64 ± 20	3
1 atm	7	130 ± 8	5
500 atm/He	7	114 ± 25	5

[1] A barotolerant marine heterotroph EP-4 (149) was grown exponentially in a glutamate-containing artificial seawater medium (147), serially diluted, and spread onto 5-cm agar plates of the same composition. The plates were incubated (20°C) at 1 atm and under 500 atm of oxyhelium (final pO_2: 0.19 atm) according to procedures described by Taylor (147, 148). Upon completion of incubation, the pressure chamber was slowly (3 hr) decompressed. Colonies were visualized by staining with iodine vapors, and then photographed and enumerated.

[2] Data are expressed as the mean and standard deviation found from "N" samples. The larger experimental error observed for the helium cultures reflects the increased difficulty in enumerating colonies (diameter 0.3–0.4 mm) against the background of numerous bubbles that formed in the agar during decompression relative to the 1-atm controls (average colony diameter 1.4 mm). Colony size differences resulted primarily from the influence of hydrostatic pressure on lag period and growth rate (147).

consistently. It is likely that the distribution of temperature- and pressure-adapted bacteria in the water column and the surface of deep-sea sediments is strongly affected by the considerable particle flux (as discussed above) from productive surface waters downward. Turner (155) has observed numbers of bacteria attached to and probably growing on the surface of fecal pellets. Many of the bacteria encountered at great depths may thus represent rather recent arrivals from surface waters. This situation is corroborated by the results of studies with natural microbial populations that, in most cases, increase their metabolic rate upon decompression (62). Most barophilic isolates have been obtained so far from the guts of deep-sea invertebrates (25, 27, 105, 170–172). These isolates are growing on relatively rich and complex media only, commonly with a 1:5 yeast extract:peptone composition at a total concentration of at least 0.1% w/v. These facts make it likely that temperature- and pressure-adapted copiotrophic rather than oligotrophic (108) bacteria mainly occur in nutrient-rich niches or, vice versa, that adaptations occur primarily in actively growing populations.

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

Effects of the specific deep-sea pressures and temperatures on bacterial metabolism have been studied with the ultimate hope of detecting unifying principles in the mechanisms by which microorganisms tolerate or adapt to deep-sea conditions. Due to the lack of deep sea–adapted isolates, general metabolic studies or more specific physiological and biochemical investigations have been conducted using natural populations or pure cultures of barotolerant

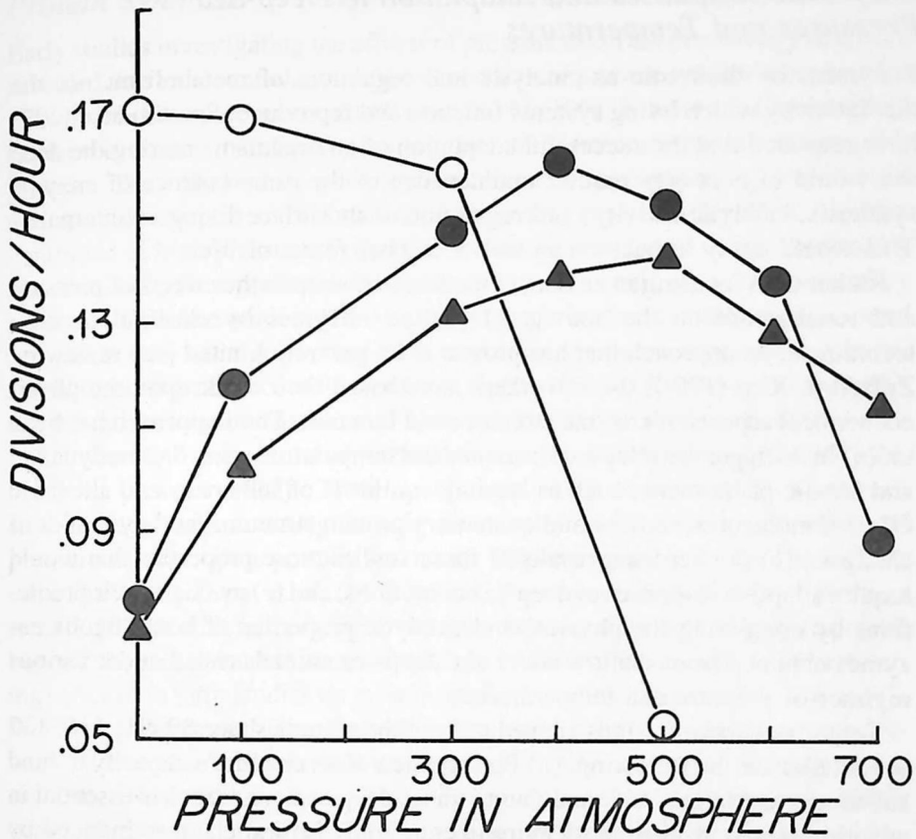


Figure 4 Growth response of a barotolerant strain (strain 40K, *circles*, isolated from 4000 m) and two barophilic strains (51A-1, *triangles;* and 72, *dots;* both isolated from 4950 m) in a peptone-yeast extract medium at 3°C. Strain 72 was isolated in the absence of decompression.

organisms, respectively. From the detailed reviewing of this work (10, 93, 95, 98, 102, 134, 174, 177) it is apparent that no clear notion exists at this time about a primary site of adaptation, except for the suggestion that processes of macromolecular biosynthesis may be more limited by pressure than processes of substrate respiration (47, 62, 64, 110, 145). Even in these latter studies the evidence is scant, largely because of their incompatibility in focus, methodology, and experimental conditions, and the metabolic variety of organisms used.

For these reasons we will limit our further discussion primarily to two recent schools of thought and experimental approach: that of P. W. Hochanchka, G. N. Somero, and colleagues, who have focused on enzymatic responses and adaptation to pressure and temperature, and that of J. V. Landau and colleagues, who have centered on detailed mechanisms of protein synthesis as the sites of pressure sensitivity, tolerance, and adaptation.

Enzymatic Responses and Adaptation to Deep-Sea Pressures and Temperatures

Enzymes, by their role as catalysts and regulators of metabolism, are the mediators by which living systems function and reproduce. Somero et al (137) have reasoned that the successful adaptation of an organism entering the deep sea would of necessity require maintenance of the same balance of enzyme synthesis, catalytic activity, and regulation as its surface-living counterparts. This would apply to bacteria as well as higher forms of life.

Rather than focusing in an encyclopedic fashion upon the effects of pressure and temperature on the activity of various enzymes by classical in vitro techniques, an approach that has proven to be severely limited [see review by ZoBell & Kim (178)], these workers have based their work upon the physicochemical aspects of enzyme structure and function. Their approach has been to: (*a*) investigate the effects of pressure and temperature upon thermodynamic and kinetic phenomena, such as binding equilibria of substrates and allosteric effector molecules, tertiary and quaternary protein structure, and dynamics of catalysis; (*b*) predict from results of these studies those properties that would require adaptive responses to deep-sea conditions; and (*c*) evaluate their predictions by comparing the physical and catalytic properties of homologous enzymes obtained from shallow water and deep-sea animals tested under various regimes of pressure and temperature.

Tentative generalizations arrived at from these studies (see 89–91, 136, 137 for details) are the following: (*a*) Preservation of an enzyme's capacity to bind substrates, cofactors, and modulators under deep-sea conditions is essential in adapting metabolism to that environment. (*b*) Structural changes induced by pressures as low as 50–100 atm may modify enzyme function sufficiently to favor selection for pressure insensitivity. It appears, in addition, that an enzyme adapted to these low pressures is also capable of maintaining proper functioning at pressures up to one order of magnitude higher. (*c*) The capacity of an enzyme to function independently of pressure is acquired at the cost of a reduction in catalytic efficiency. A low temperature, high pressure–adapted enzyme would, therefore, function at low pressure less efficiently in terms of catalytic rate than would a low temperature, low pressure–adapted homolog. The converse would also be true, the low pressure–adapted enzyme being less efficient at elevated pressure. Thus, in terms of optimal metabolic activity, a segregation of organisms would occur within the water column in accordance with the pressure adaption of key enzymes. (*d*) In view of the strong influences of pH, ionic strength, and ionic composition upon enzyme structure and function and, therefore, the effects of temperature and pressure upon measured enzyme properties, it is crucial that assay conditions simulate the intracellular milieu as closely as possible.

Protein Synthesis at Deep-Sea Pressures and Temperatures

Early studies investigating the effects of pressure upon the physiology of *E. coli* revealed that an essentially complete cessation of protein synthesis occurred at growth-inhibiting pressures of 680 atm (80, 81, 109). Schwarz & Landau (126) began the series of studies for tracing the site of pressure inhibition upon this process. They showed in whole-cell studies that the supporting activities of amino acid transport and formation of the amino acyl–transfer RNA complex continued at high pressure. Polysome formation was found to be inhibited by pressure, but, when preformed at 1 atm, polysomes could not be dissociated by pressure. Cell-free studies (127) using puromycin revealed that peptide-bond formation was not the limiting step in protein synthesis, but rather was the result of an interference with amino acyl–transfer RNA binding to the polysome, an observation that was in agreement with the results of others (1).

Schwarz & Landau further postulated that translocation may also be inhibited by pressure (127). Their conclusions were confirmed in whole-cell studies (113), substantiating the role of ribosome dysfunction for the observed pressure effects upon protein synthesis. Expanding their studies to include other bacteria, Pope et al (112) found *Pseudomonas fluorescens* to be a remarkably barotolerant organism that was capable of synthesizing protein well beyond the pressures that were completely inhibitory for *E. coli*. In an interesting series of in vitro studies on protein synthesis, using extracts of *E. coli* and *P. fluorescens* separated into their ribosomal and soluble cytoplasmic constituents, it was clearly shown that the observed *P. fluorescens* barotolerance was associated with the ribosome (112). The functioning of the required soluble components was completely unaffected by pressure, independent of source. Extending this line of study one step further revealed that barotolerant protein synthesis was associated with the 30S ribosomal subunit (83, 135).

Soon thereafter a very interesting observation was made, showing that the genetic alteration of the 30S ribosomal subunit that confers streptomycin resistance to *E. coli* (K-12^r) was also sufficient to impart the capability of barotolerant protein synthesis (111). Association of barotolerance with the 30S ribosomal subunit appeared to be a feature of a number of organisms (111), leading Landau & Pope (82) to postulate in a broad sense the importance of ribosomal structure and function for an organism's ability to synthesize protein under high pressure.

This approach has also been successfully applied in investigating the mechanism of low temperature growth inhibition of *E. coli* and other organisms. Results of a series of in vivo and in vitro experiments suggest that subminimal temperatures prevent the initiation of translation, resulting in the accumulation of 70S particles in the cytoplasm and the subsequent termination of protein synthesis (14). Similar polysome and protein dysfunctions have been

observed or inferred in a number of instances [as reviewed by Inniss & Ingraham (48) and Jaenicke (50)].

The specific reasons why proteins and other cytoplasmic elements fail at high pressure and low temperatures in one organism and not in another remain unexplained. In other words, the mechanisms by which deep-sea microbes have adapted to this environment are still essentially unknown. The kind of investigations discussed above, namely the integration of predictive physicochemical principles with systematic laboratory study, can be expected to unravel the nature of barophily and the mechanisms of adaptation to the deep-sea conditions.

The recent acquisition of obligate barophilic and psychrophilic microorganisms should remove the major barrier to such direct studies of adaptation to deep-sea pressures and temperature. Through radioisotope experiments employing established techniques of subcellular fractionation (23, 118), it should be possible to identify the major metabolic functions that fail below optimal hydrostatic pressures. This will provide the foundation for detailed and systematic biochemical study into the nature of barophily.

DEEP-SEA HYDROTHERMAL VENTS

The notion that life in the deep sea is characterized by its remoteness from the input of energy and nutrients (123, 157) and by a low and constant temperature had to be qualified in 1977 when dense and thriving populations of invertebrates were discovered at hydrothermal vents at about 2600 m depth (4, 22, 87). It appeared that various types of bacteria, taking the place of photosynthetic primary producers of organic carbon, use reduced inorganic constituents of the emitted hydrothermic fluid as a source of geothermal energy for chemosynthesis (59, 72).

Although most of the chemosynthetic processes so far found at these vents are aerobic, i.e. requiring photosynthetically generated free oxygen, the term *primary* production is useful and justified on the grounds that it is defined by the source and not the sink of energy (52). In *secondary* production, organic carbon is recycled, and the source of energy is biogenic. Geothermic energy in the form of reduced inorganic compounds also occurs in terrestrial hot springs. However, in the presence of photosynthetic processes, microbial chemosynthesis appears to contribute insignificantly to total primary production. It is the most surprising part of the discovery of these rich animal population at the vents that chemosynthesis indeed appears, under the particular conditions, to provide a food source at least as efficient as photosynthetic systems, if not more so considering the very direct transfer of intermediate products through highly developed symbioses as a shortcut food chain (see below).

Geothermal Sources of Energy

The deep-sea vents were discovered as the result of a systematic search for active volcanism at submarine spreading centers, as predicted by the theory of plate tectonics (30, 86, 119, 163). At these zones, extending for about 25,000 miles throughout the Atlantic, Pacific, and Indian Oceans, freshly extruded lava contracts upon cooling and allows seawater to penetrate several kilometers downward into the newly formed crust. Reacting with basaltic rock at high pressures and temperatures exceeding 350°C, the seawater is transformed into an acidic and highly reduced "hydrothermal fluid" enriched in metals, hydrogen sulfide, and molecular hydrogen. The rising hydrothermal fluid comes in contact with oxygen, either when mixing with seawater in the porous subsurface layer of pillow lava and emerging as "warm" vents (8–23°C) at rates of 1–2 cm sec^{-1}, or when discharging directly into bottom seawater—without prior mixing—as "hot" vents (approximately 350°C) at rates of about 2 m sec^{-1}. Warm-water vents may or may not contain dissolved oxygen, whereas hot-water vents are always anoxic. Most warm-vent waters so far analyzed exhibited significant chemical differences. The major reactions, however, follow the scheme given in Figure 5.

Leaching of sulfur from basalt results in an enrichment of the hydrothermal fluid in sulfide beyond the amount corresponding to the original sulfate content of seawater. The highly variable sulfide concentration of vent waters (10–160 $\mu mol\ liter^{-1}$) depends on the enroute deposition of metal sulfides. Carbon is also enriched and occurs in both its most oxidized and reduced forms. The generally assumed geothermal origin of methane and hydrogen (85, 161) in hydrothermal fluid is somewhat challenged by indications of bacterial methane production (6, 68). Magnesium is entirely removed from seawater during the passage through the crust by a temperature-controlled reaction (29), a fact used for the calculation of mixing quotients in the analysis of nonreactive constituents of hydrothermal fluid in seawater. The enrichment of calcium by two- to four-fold leads to the immediate precipitation of anhydrite (anhydrous calcium sulfate) upon mixing of hydrothermal fluid with seawater. This precipitation may result in the clogging and relocation of warm vents and the formation of "chimneys" around the openings of hot vents.

Similarly the precipitation of iron and manganese sulfides affects the flow and the chemical composition of vent waters. The type of minerals found at the spreading centers depends on the mode of their formation and solubility under the prevailing conditions [for a recent overview see Mottl (103)]. The most dramatic enrichment is that of manganese, amounting to several orders of magnitude. The manganese content of seawater, in excess of the caused by weathering and land runoff, has been used to estimate that the entire amount of global seawater circulates through the crust once every 8 million years. This rate equals about 0.5% of the total flow of rivers into the world's oceans (31).

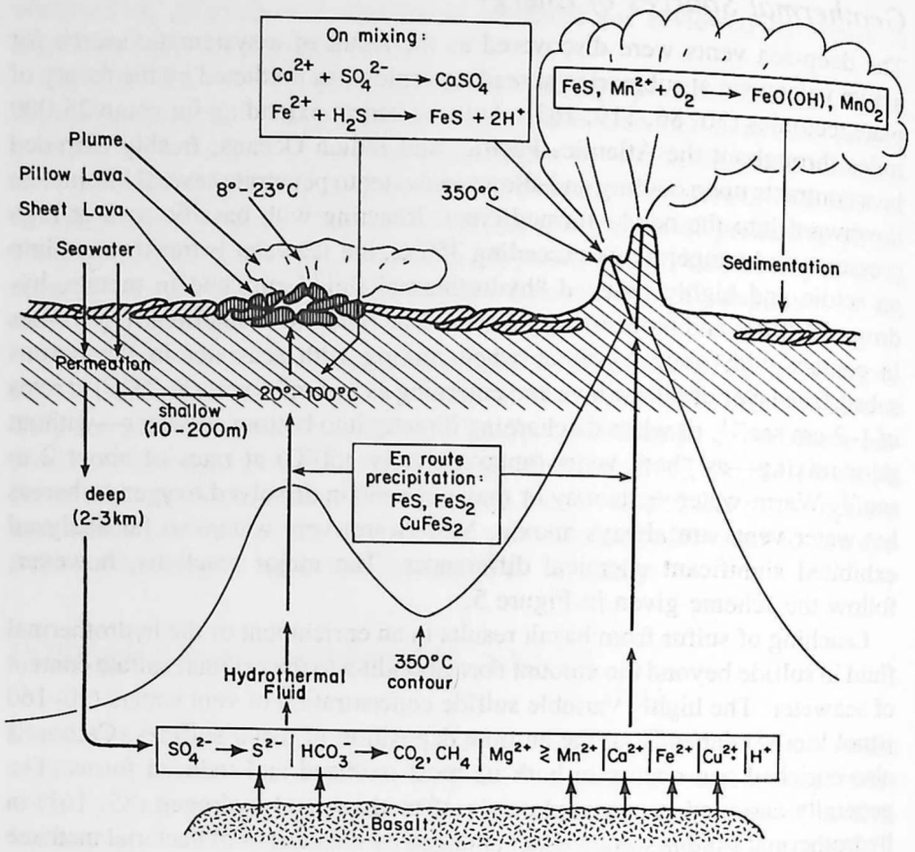


Figure 5 The major geochemical reactions occurring during the circulation of seawater through the earth's crust at the ocean floor spreading centers. The two major types of vents are indicated.

Occurrence and Role of Microorganisms

For work at the relatively small area of the deep-sea vents, in the range of square meters, the research submersible *Alvin* was indispensible. Accuracy, frequency, and replication of sampling are limited. Nevertheless, there is now sufficient direct and circumstantial evidence for microbial chemosynthetic growth and activity at three major sites of the vent systems: (*a*) in bacterial cell suspensions contained in the emitted warm-vent waters; (*b*) in microbial mats growing on various surfaces in the immediate vent surroundings that were exposed to the plume of emitted vent waters; and (*c*) in symbiotic associations between chemosynthetic procaryotes and the most abundant types of vent invertebrates.

The turbid waters emitted from several of the warm vents at the Galapagos Rift site (0°47'N, 86°08'W) contained 10^6–10^9 bacterial cells ml^{-1} (22, 52, 59) as determined by epifluorescence microscopy of acridine orange–stained

samples (24). The large differences in cell numbers in these samples were the result of irregular mixing of the emitted vent water with seawater. Normalization of these numbers on the basis of the magnesium content as a mixing index (see above) has thus far not been made. The concentration of adenosine 5'-triphosphate (ATP) was determined as an approximate measure of biomass and the guanosine 5'-triphosphate:adenosine 5'-triphosphate (GTP:ATP) ratio as an estimate of the rate of cellular biosynthesis (70, 71).

As shown in Table 2, the ATP concentrations were found to be two- to three-fold higher in vent waters than in the photosynthetically productive surface waters at the same site. The emitted turbid waters also contained flocculant particles visible from the submersible (52). Microscopy revealed them to consist primarily of aggregated bacterial cells (59), suggesting growth of bacterial mats within the (inaccessible) vent system and their dislodgement by the extruding waters. A particular fish, only found in association with the Galapagos Rift vents, is commonly observed head-down in vent openings, most likely feeding on microbial mat material. If this observation can be confirmed, it will constitute an extraordinarily short food chain. Warm-water pockets (20–25°C) at one vent site (21°09'N, 109°15'W) were slightly turbid and contained 10^6–10^7 cells ml^{-1}. A new genus of zooplankton (39) was described. The animal was found in large numbers feeding on these bacterial cell suspensions at the vent-seawater interface. Bacteriological work conducted at this vent site on a recent cruise is in preparation.

Rates of CO_2 fixation in turbid vent water were measured in situ and in the ship's laboratory (53). Sets of 200-ml syringes, precharged with ^{14}C-labeled bicarbonate, were filled in a vent plume; some were left near the collection site for incubation, while others were returned to the surface for incubation at 1 atm and controlled temperature. Preliminary data show a CO_2-incorporation rate of 1 nmol $liter^{-1}$ day^{-1} in situ at 3°C (outside of the plume) as well as at 1 atm and

Table 2 Microbial nucleotides in samples from the Galapagos Rift site

Sample	ATP	A_T	GTP:ATP
Surface seawater[a] (50 m)	130 ± 32	340 ± 112	0.16 ± 0.08
Deep seawater[a] (2400 m)	1.7 ± 0.3 ng/liter	3.4 ± 0.3 ng/liter	0.075
"Garden of Eden" vent[a] (2500 m)	491 ± 151	1494 ± 553	0.86 ± 0.17
"Garden of Eden" vent[b] (2500 m)	1943 ± 1143 ng/g	4248 ± 2031 ng/g	0.89 ± 0.35

Data from (72); ATP = adenosine 5'-triphosphate, A_T = total adenylates, GTP:ATP = ratio of guanosine 5'-triphosphate to adenosine 5'-triphosphate.
[a] Filtered
[b] Settled particles

3°C, and a rate of about 100 nmol liter^{-1} day^{-1} at 25°C and 1 atm (53). These data indicate barotolerant and mesophilic growth characteristics of the microbial populations emitted from the vents.

Furthermore, the addition of thiosulfate (1.0 mM) increased the rate of CO_2 fixation considerably. Experiments with the above-described pressure-retaining sampling system (64) confirmed the nonbarophilic growth responses (H. W. Jannasch, unpublished observations). In general, the CO_2-assimilation in emitted vent waters, freshly mixed with oxygenated seawater of 3°C, was minor compared to the chemosynthetic biomass production within the subsurface vent system prior to emission. The result of isolation attempts as well as the measured uptake of acetate and glucose indicated the presence of mixotrophic or facultatively chemolithotrophic bacteria in the emitted vent waters (122, 156).

Work on the isolation of bacteria from vent waters focused first on sulfur oxidizers because of the predominance of reduced sulfur compounds in vent waters. From more than 200 isolates, a number of acid-producing, obligately chemolithotrophic as well as base (polythionate)-producing, facultatively chemolithotrophic strains were described (122). Of the former, the genus *Thiomicrospira* appeared predominant. A representative strain (L-12) was characterized (121) by microaerophilic growth, a high sulfide tolerance (up to 2 mM), a temperature optimum at 25°C, and an almost linear decrease in CO_2 fixation activity between 100 and 500 atm. At the in situ pressure of its origin (260 atm), this organism retains about 75% of its 1-atm activity. The 5S ribosomal RNA (rRNA) sequence of this strain was significantly different from the type strain of the species *Thiomicrospira pelophila* (77, 140). With more recent isolates, slightly higher temperature optima have been found (26°–33°C), and doubling times as short as 1 hour have been observed (56).

Most of these organisms could also be isolated from the heavy microbial mats that cover all types of surfaces in the immediate surroundings of the vents where they are exposed to the plume. A survey of morphological types of organisms lists six predominant forms (61): (*a*) Unicellular cocci were covering or deeply embedded in iron-manganese oxide deposits. Strains of manganese-oxidizing bacteria were isolated and described (32). (*b*) *Hyphomonas-Hyphomicrobium*–type cells with extremely long stalks were most commonly observed. Several new *Hyphomonas* isolates were described by Weiner et al (160). (*c*) Cyanobacteria-like trichomes morphologically undistinguishable from *Calothrix* were found frequently, some containing basal heterocysts. These organisms might represent chemosynthetic counterparts to *Calothrix* analogous to the *Oscillatoria/Beggiatoa* pair (61). (*d*) Up to 15% of the cells within the mats contained extensive cytoplasmatic membrane systems. These organisms were morphologically identified as Type I methylotrophic bacteria by R. Whittenbury (personal communication); this was physiologically confirmed by successful isolations (R. Hanson, personal communication). (*e*) Attached filaments resembling *Thiothrix* or *Leucothrix* were the most commonly observed organisms at all vent sites so far studied. (*f*) Nonattached *Beggiatoa*-like filaments were first observed on artifical surface material (slate) deposited at the Galapagos Rift vent site for a recovery after 10.5 months (61). From one sample an anaerobic free-living spirochete was isolated after its observation by phase microscopy in microbial mat material (41).

The presence of thermophilic bacteria in samples from deep-sea hydrothermal vents was indicated by the fact that rates of ^{3}H-adenine incorporation into DNA and RNA (70) were unusually high when incubated at 90°C. The rate of organic carbon production (extrapolated from measurements of DNA synthesis) at 90°C was equivalent to 19 μg C g^{-1} hr^{-1}, which corresponds to a specific growth rate of 0.55 hr^{-1} (D. M. Karl, unpublished observations). In samples of hot-vent water enriched with a number of inorganic salts and formate, the formation of CH_4, H_2, and CO was observed after incubation at 100 ± 2°C (6). From deposits at the base of a "hot smoker" chimney, Jones et al (68) isolated and described a new species of the genus *Methanococcus* that grows optimally at 86°C with a doubling time of 26 min. Instead of tetraethers, common in the archaebacteria, the polar membrane lipid of this species was found to be primarily (95%) a unique macrocyclic glycerol diether (21).

From terrestrial and shallow marine hot springs a number of extremely thermophilic bacteria have recently been isolated (13, 38, 173), optimal growth in one case taking place at 105°C (141). Such conditions require elevated pressures to keep water in liquid state, and it is expected that similar organisms will be isolated from the deep-sea vents. (The boiling point of water at a depth of 2500 m is about 460°C). Growth at 250°C of a mixed microbial population in water collected from a "hot smoker" was reported (5) in a study that, at this time, still awaits confirmation.

The general notion of sulfur-based chemosynthesis as a major source of food for filter-feeding bivalves generated research with the practical aim of combining bacterial biomass production from industrial sulfide waste sources with aquaculture (51, 59). Growth and harvesting of colorless sulfur bacteria in various types of continuous flow reactors as well as the ingestion and digestion of this bacterial food by common shellfish is presently being studied in our laboratories.

Chemolithotrophic Symbionts

It had been suspected early that the density of bacterial cells near the vents could not completely account for the unusual abundance and activity of the two major species of invertebrates, the clams *(Calyptogena magnifica)* (9) and the vestimentiferan tube worms *(Riftia pachyptila)* (67). From histological and enzymatic studies it is now established with reasonable certainty that the major

and most efficient transfer of chemosynthetically produced organic carbon to these invertebrates takes place by a newly discovered type of symbiosis (16, 17, 33, 34). There is no evidence that the densely clustered populations of these two species receive a substantial food supply from a photosynthetic source.

The 2.5-m-long and 5-cm-wide tube worms are mouthless and gutless. Their body cavity is filled with a spongy tissue, the "trophosome", that may represent over 50% of the animal's wet weight. The symbiotic cells within this tissue do not contain internal membrane–bound organelles, nor do they possess gram-negative–type cell walls or a non–membrane-bound nuclear region (17). The presence of procaryotic cells was further established by positive lipopolysaccharide tests (17, 159). In the trophosome the Calvin-cycle enzymes ribulose biphosphate carboxylase (RuBPC) and ribulose 5-phosphate kinase (Ru5PC) were found (33), providing additional evidence for microbial reduction of CO_2. Enzymes involved in the ATP-producing transformation of sulfur compounds were: rhodanese, which cleaves thiosulfate into sulfite and sulfur and catalyzes the transfer of sulfur from thiosulfate to glutathione (143); ATP sulfurylase, the enzyme that activates sulfate by the formation of adenosine 5'-phosphosulfate (APS) (106); and APS reductase, which catalyzes the reversible formation of APS from AMP and sulfide (104, 107). Smaller gutless Pogonophora were previously found in many marine, organic-rich and predominantly reduced habitats apart from the vents. Once believed to absorb dissolved organic matter through the epidermis, it has now been shown that chemosynthetic symbiosis is responsible for at least a large part of the nutrition of these animals (138, 139).

Microbiological work on the symbionts of *R. pachyptila* has met with difficulties in isolation. Scanning electron microsocpy has revealed large spherical cells (3.5 μm diameter) at densities of up to 10^9 ml^{-1} and a variety of short rods (52). It is unclear at this point whether this morphological heterogeneity represents polymorphism of a single symbiont or an actual diversity of morphological and physiological types. Isolates obtained thus far from high dilutions of fresh trophosome material do not answer this question with any certainty (56). Only one isolate resembles the above large spherical cells. However, it appears to be a hydrogen-oxidizing organism and is not stimulated by reduced sulfur compounds. Other isolates are growing on reduced sulfur compounds but are not obligately chemolithotrophic.

It can be argued that auxotrophic-mixotrophic organisms can be more efficiently regulated by the host while still fixing sufficient amounts of CO_2. Determining the base ratio of frozen trophosome material resulted in a biphasic melting curve indicating, in addition to some DNA of a typical invertebrate composition, a major procaryotic component containing approximately 60 mol% guanine plus cytosine (56). This ratio is typical for some species of the genus *Thiobacillus* (78). The actual chances for the isolation of a culturable symbiont will be indicated by the results of ongoing studies for determining the genome size of the procaryotic trophosome component.

The symbiosis between chemosynthetic procaryotes and the gutless worms implies the transport of both free oxygen and hydrogen sulfide from the gills to the trophosome by an efficient and specially equipped blood system. Particularly high affinities and carrying capacities of the extracellular hemoglobin for oxygen were described (2, 150, 167). It appears that a special sulfide-binding process is responsible for preventing respiratory poisoning (3, 114).

Following the general assumption that chemosynthetic bacteria might inhabit the gill surface of the fast-growing (116, 153) giant white clams, microscopic studies revealed procaryotes as endosymbionts within the gill tissue (16). Enzymatic evidence of their chemolithotrophic activity has been presented and a successful search undertaken to find similar symbioses in bivalves that typically live in sulfide-containing marine habitats such as shelf sediments, estuaries, and marshes (17, 35). Although no isolates have been obtained to date, work on chemosynthetic symbiosis in bivalves carries higher promises than that on the Pogonophora: work is not limited by the problems of deep-sea operations and by the difficulty of experimenting with live deep-sea animals in the laboratory.

Using the comparative analysis of 5S ribosomal RNA (rRNA) nucleotide sequences, Stahl et al (140) studied the thus-far unculturable symbionts of *Riftia, Calyptogena,* and a nonvent bivalve, *Solemya*. This approach permits the assessment of the phylogenetic position of the symbiotic procaryotes and thereby their fundamental physiological capabilities in the absence of growth. The symbiont ribosomal contribution to *Riftia* trophosomal tissue was about 75%. The procaryotic contribution to the gill tissue of *Calyptogena* and *Solemya* was 75% and 20% respectively. These first results suggest that there is just one type of symbiont per animal species, implying the above mentioned polymorphism.

The geothermic diversity of vent types thus far observed precludes quantitative statements on the chemosynthetic input of organic carbon into the deep sea at these particular sites. For instance, an entirely new situation was found at a recently discovered vent site (Guaymas Basin, Gulf of California) where hydrothermal discharges are overlayed by young sediments of an actively depositing (> 1 m per 1000 years) mixture of terrigenous and pelagic debris (88). The oxygen concentration in the bottom water is low (0.6–0.7 ml liter^{-1}) and the organism-rich diatomaceous mud (2–4% organic carbon) appears to be anoxic slightly below the sediment surface, with an H_2S concentration of 1.5 mmol liter^{-1} at 1 m depth. High concentrations of ammonia (25 mmol liter^{-1}) (J. M. Edmond, personal communication) raise the possibility that a chemosynthetic production of organic carbon at this site may be partly based on nitrification. The photosynthetic diatomaceous input at this site is believed to be involved in the hydrothermic, or possibly biological, formation of gasoline-type hydrocarbons that have been detected in the sediments (133). Thick layers of *Beggiatoa*-like filaments with a cell width of up to 100 μm were observed

(52), but so far obtained only as fixed-sample material. A special biological study of this site is in the planning stage.

CONCLUDING REMARKS

The preceding discussion of present-day approaches in deep-sea microbiology reveals a lack of compatibility between fundamental but relatively simple field studies on growth of natural populations and laboratory experiments with pure cultures under well-defined conditions. Results of the former are often characterized by logistic constraints of work at sea, e.g. insufficient data to permit statistical treatment. Laboratory studies, on the other hand, are done with organisms removed from their natural habitat for some time and grown in media not reflecting the in situ nutritional conditions. Advancing technology is beginning to close this gap, especially with instrumentation facilitating isolation and growth of deep-sea bacteria in the absence of temperature and pressure changes.

In addition to low temperature–adapted bacteria from the deep sea, barophilic growth characteristics have now been unequivocally demonstrated. Such pressure-adapted organisms can be isolated with relative ease and grown in the laboratory. Their availability will greatly facilitate studies of the biochemical and molecular basis of barophilism in the coming years. The observed marginal pressure effects in the range of 100–600 atm will be much more difficult to identify than those in the range of 600–1100 atm; yet the latter applies to only 0.1% of the deep sea by volume.

Studies on the deep-sea hydrothermal vents have broadened our perspectives of chemosynthetic bacteria as primary producers. Progress in the work on the various microbial processes at these vents is somewhat constrained by the relatively rare opportunities to obtain fresh sample material. The discovery, however, that the newly found symbiosis between chemolithotrophic procaryotes and certain vent invertebrates can also be detected in bivalves that typically inhabit strongly reduced marine sediments of shallow coastal waters does indeed facilitate experimental approaches and promise rapid progress in this area. In hindsight it has turned out that the detour via deep-sea observations was necessary in order to uncover this entirely new microbially supported ecosystem and procaryote-eucaryote symbiotic interaction.

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Literature Cited

1. Arnold, R. M., Albright, L. J. 1971. *Biophys. Acta* 238:347–54
2. Arp, A. G., Childress, J. J. 1981. *Science* 213:342–44
3. Arp, A. G., Childress, J. J. 1983. *Science* 219:295–97
4. Ballard, R. D. 1977. *Oceanus* 20:35–44
5. Baross, J. A., Deming, J. W. 1983. *Nature* 303:423–26
6. Baross, J. A., Lilley, M. D., Gordon, L. I. 1982. *Nature* 298:366–68
7. Baross, J. A., Morita, R. Y. 1978. In *Microbial Life in Extreme Environments*, ed. J. D. Kishner, pp. 9–71. New York: Academic
8. Benecke, W. 1933. *Abderh. Hand. Biol. Arbeitsmeth.*, IX Abt., Teil 5:717–854
9. Boss, K. J., Turner, R. D. 1980. *Malacologia* 20:161–94
10. Brauer, F. W., ed. 1972. *North Carolina Sea Grant Program*. Chapel Hill, NC: Univ. N. Carolina. 428 pp.
11. Brauer, R. W., Hogan, P. M., Hugon, M., Macdonald, A. G., Miller, K. W. 1982. *Undersea Biomed. Res.* 9:353–96
12. Brewer, P. G., Nozaki, Y., Spencer, D. W., Fleer, A. P. 1980. *J. Mar. Res.* 38:703–28
13. Brock, T. D. 1978. *Thermophilic Microorganisms and Life at High Temperatures*. New York: Springer-Verlag
14. Broeze, R. J., Solomon, C. J., Pope, D. H. 1978. *J. Bacteriol.* 134:861–74
15. Bruland, K. W., Silver, M. W. 1981. *Mar. Biol.* 63:295–300
16. Cavanaugh, C. M. 1983. *Nature* 302:58–61
17. Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W., Waterbury, J. B. 1981. *Science* 213:340–41
18. Certes, A. 1884. *C. R. Acad. Sci. Paris* 98:690–93
19. Certes, A. 1884. *C. R. Soc. Biol.* 36:220–22
20. Colwell, R. R., Tabor, P. S. 1982. In *The Dynamic Environment of the Ocean Floor*, ed. K. S. Fanning, F. T. Manheim, pp. 149–60. Lexington, MA: D. C. Heath
21. Comita, P. B., Gagosian, R. B. 1984. *Science*. 222:1329–31
22. Corliss, J. B., Dymond, J., Gordon, L. I., Edmond, J. M., von Herzen, R. P., et al. 1979. *Science* 203:1073–83
23. Cuhel, R. L., Taylor, C. D., Jannasch, H. W. 1981. *Arch. Microbiol.* 130:1–7
24. Daley, R. J., Hobbie, J. E. 1975. *Limnol. Oceanogr.* 20:875–82
25. Deming, J. W., Colwell, R. R. 1982. *Appl. Environ. Microbiol.* 44:1222–30
26. Deming, J. W., Colwell, R. R. 1982. *BioScience* 31:507–11
27. Deming, J. W., Tabor, P. S., Colwell, R. R. 1981. *Microb. Ecol.* 7:85–94
28. Dietz, A. S., Yayanos, A. A. 1978. *Appl. Environ. Microbiol.* 36:966–68
29. Edmond, J. M., Measures, C., McDuff, R. E., Chan, L. H., Collier, R., et al. 1979. *Earth Planet. Sci. Lett.* 46:1–18
30. Edmond, J. M., von Damm, K. L., McDuff, R. E., Measures, C. I. 1982. *Nature* 297:187–91
31. Edmond, J. M., von Damm, K. 1983. *Sci. Am.* 248:78–93
32. Ehrlich, H. 1983. *Ecol. Bull.* 35:357–66
33. Felbeck, H. 1981. *Science* 213:336–38
34. Felbeck, H. 1983. *J. Comp. Physiol.* 152:3–11
35. Felbeck, H., Childress, J. J., Somero, G. N. 1981. *Nature* 293:291–93
36. Fischer, B. 1886. *Z. Hyg.* 1:421–64
37. Fischer, B. 1894. *Centralbl. Bakteriol.* 15:657–66
38. Fischer, F., Zillig, W., Stetter, K. O., Schreiber, G. 1983. *Nature* 301:511–13
39. Fleminger, A. 1983. *Proc. Biol. Soc. Wash.* 96:605–22
40. Haedrich, R. L., Rowe, G. T. 1977. *Nature* 29:141–42
41. Harwood, C. S., Jannasch, H. W., Canale-Parole, E. 1982. *Appl. Environ. Microbiol.* 44:234–37
42. Hinga, K. R., Sieburth, J. McN., Heath, G. R. 1979. *J. Mar. Res*, 37:557–79
43. Honjo, S. 1978. *J. Mar. Res.* 36:469–92
44. Honjo, S., Manganini, S. J., Cole, J. J. 1982. *Deep-Sea Res.* 29:609–25
45. Huxley, T. H. 1868. *Q. J. Microscop. Sci. (NS)* 8:203–12
46. Ingraham, J. L. 1958. *J. Bacteriol.* 75:75–80
47. Ingraham, J. L., Stokes, J. L. 1959. *Bacteriol. Rev.* 23:97–108
48. Inniss, W. E., Ingraham, J. L. 1978. See Ref. 7, pp. 73–104
49. Isaacs, J. D. 1969. *Sci. Am.* 221:146–62
50. Jaenicke, R. 1981. *Ann. Rev. Biophys. Bioeng.* 10:1–67
51. Jannasch, H. W. 1979. *Oceanus* 22:59–63
52. Jannasch, H. W. 1984. See Ref. 119, pp. 677–709
53. Jannasch, H. W. 1984. In *The Microbe 1984*, Part III, *Prokaryotes and Eukaryotes*, ed. D. P. Kelly, N. G. Carr, pp. 97–122. Soc. Gen. Microbiol. Symp., Cambridge: Univ. Press
54. Jannasch, H. W., Cuhel, R. L., Wirsen, C. O., Taylor, C. D. 1980. *Deep-Sea Res.* 27:867–72

55. Jannasch, H. W., Eimhjellen, K., Wirsen, C. O., Farmanfarmaian, A. 1971. *Science* 672–75
56. Jannasch, H. W., Nelson, D. C. 1984. In *Current Perspectives in Microbial Ecology*, ed. C. A. Reddy, M. J. Klug, pp. 170–76. Washington DC: Am. Soc. Microbiol. In press
57. Jannasch, H. W., Wirsen, C. O. 1973. *Science* 180:641–43
58. Jannasch, H. W., Wirsen, C. O. 1977. *Appl. Environ. Microbiol.* 33:642–46
59. Jannasch, H. W., Wirsen, C. O. 1979. *BioScience* 29:592–98
60. Jannasch, H. W., Wirsen, C. O. 1980. In *Biogeochimie de la Matiere Organique a l'Interface Eau-Sediment Marin. Actes Colloq. C. N. R. S.*, ed. R. Roumas, 293:285–90
61. Jannasch, H. W., Wirsen, C. O. 1981. *Appl. Environ. Microbiol.* 41:528–38
62. Jannasch, H. W., Wirsen, C. O. 1982. *Appl. Environ. Microbiol.* 43:1116–24
63. Jannasch, H. W., Wirsen, C. O. 1983. In *Deep-Sea Biology*, ed. G. T. Rowe, pp. 231–59. New York: Wiley
64. Jannasch, H. W., Wirsen, C. O., Taylor, C. D. 1976. *Appl. Environ. Microbiol.* 32:360–67
65. Jannasch, H. W., Wirsen, C. O., Taylor, C. D. 1982. *Science* 216:1315–17
66. Jannasch, H. W., Wirsen, C. O., Winget, C. L. 1973. *Deep-Sea Res.* 20:661–64
67. Jones, M. L. 1980. *Proc. Biol. Soc. Wash.* 93:1295–313
68. Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R., Wolfe, R. S. 1983. *Arch. Microbiol.* 136:254–61
69. Jørgensen, B. B. 1983. In *The Major Biogeochemical Cycles and Their Interactions*, ed. B. Bolin, R. B. Cook, pp. 477–509. Chichester, UK: Wiley
70. Karl, D. M. 1980. *Microbiol. Rev.* 44:739–96
71. Karl, D. M. 1981. *Appl. Environ. Microbiol.* 42:802–10
72. Karl, D. M., Wirsen, C. O., Jannasch, H. W. 1980. *Science* 207:1345–47
73. Kinne, O. 1972. In *Marine Ecology*, ed. O. Kinne, 1:1323–450. London: Wiley
74. Koch, R. 1881. *Mitt. Kaiser. Gesundheitsamt* 1:1–48
75. Komar, P. D., Morse, A. P., Small, L. F., Fowler, S. W. 1981. *Limnol. Oceanogr.* 26:172–80
76. Kriss, A. E. 1962. *Marine Microbiology (Deep Sea)*. NY: Wiley Interscience
77. Kuenen, J. G., Veldkamp, H. 1972. *Antonie van Leeuwenhoek* 38:241–56
78. Kuenen, J. G., Yuovinen, O. H. 1981. In *The Prokaryotes*, ed. M. P. Stolp, H. Trüper, A. Balows, H. G. Schlegel, pp. 1023–36. Berlin: Springer-Verlag
79. Landau, J. V. 1970. In *High Pressure Effects on Cellular Processes*, ed. A. M. Zimmerman, pp. 45–70. New York: Academic
80. Landau, J. M. 1966. *Science* 153:1273–74
81. Landau, J. M. 1967. *Biochim. Biophys. Acta* 149:506–12
82. Landau, J. V., Pope, D. H. 1980. *Adv. Aquat. Microbiol.* 2:49–76
83. Landau, J. V., Smith, W. P., Pope, D. H. 1977. *J. Bacteriol.* 130:154–59
84. Lee, C., Bada, L. 1975. *Earth Planet. Sci. Lett.* 26:61–68
85. Lilley, M. D., de Angelis, M. A., Gordon, L. I. 1982. *Nature* 300:48–50
86. Lister, C. R. B. 1977. *Tectonophysics* 37:203–18
87. Lonsdale, P. F. 1977. *Deep-Sea Res.* 24:857–63
88. Lonsdale, P. F., Bischoff, J. L., Burns, V. M., Kastner, M., Sweeney, R. E. 1980. *Earth Planet. Sci. Lett.* 49:8–20
89. Low, P. S., Bada, J. L., Somero, G. N. 1973. *Proc. Natl. Acad. Sci. USA* 70:430–43
90. Low, P. S., Somero, G. N. 1974. *Comp. Biochem. Physiol. B.* 49:307–12
91. Low, P. S., Somero, G. N. 1975. *Comp. Biochem. Physiol. B.* 52:67–74
92. Madin, L. P. 1982. *Mar. Biol.* 67:39–45
93. Marquis, R. E. 1976. *Adv. Microbiol. Physiol.* 14:158–241
94. Marquis, R. E. 1982. *BioScience* 32:267–71
95. Marquis, R. E., Matsumura, P. 1978. In *Microbial Life in Extreme Environments*, ed. D. J. Kushner, pp. 105–58. New York: Academic
96. Deleted in proof
97. Marquis, R. E., Thom, S. R., Crookshank, C. A. 1978. *Undersea Biomed. Res.* 5:189–98
98. Morita, R. Y. 1972. In *Marine Ecology*, ed. O. Kinne, 1:1361–88. New York: Wiley Interscience
99. Morita, R. Y. 1970. In *Methods in Microbiology*, ed. J. R. Norris, D. W. Ribbons, 2:243–57. New York: Academic
100. Morita, R. Y. 1970. *Can. J. Microbiol.* 26:1375–85
101. Morita, R. Y. 1975. *Bacteriol. Rev.* 39:144–67
102. Morita, R. Y. 1976. In *The Survival of Vegetative Microbes, 26th Symp. Soc. Gen. Microbiol.*, ed. T. Gray, J. R. Postgate, pp. 279–98. Cambridge Univ. Press
103. Mottl, M. J. 1984. See Ref. 119, pp. 199–224
104. Muraoka, J. S. 1971. *Ocean Industry.* Feb. 1971, pp. 21–23
105. Ohwada, K., Tabor, P. S., Colwell, R. R. 1980. *Appl. Environ. Microbiol.* 40: 746–55
106. Peck, H. D. 1962. *Bacteriol. Rev.* 26:67–94
107. Peck, H. D., Deacon, T. E., Davidson, J. T. 1965. *Biochim. Biophys. Acta* 96:429–46
108. Poindexter, J. S. 1981. *Adv. Microb. Ecol.* 5:67–93
109. Pollard, E. C., Weller, P. K. 1966. *Biochim. Biophys. Acta* 112:573–80
110. Pope, D. H., Berger, L. R. 1973. *Arch. Mikrobiol.* 93:367–70
111. Pope, D. H., Smith, W. P., Orgrinc, M. A., Landau, J. V. 1976. *Appl. Environ. Microbiol.* 31:1001–2
112. Pope, D. H., Smith, W. P., Schwarz, R. W., Landau, J. V. 1975. *J. Bacteriol.* 121:664–69
113. Pope, D. H., Connors, N. T., Landau, J. V. 1975. *J. Bacteriol.* 121:753–58
114. Powell, M. A., Somero, G. N. 1983. *Science* 219:297–99
115. Rehbock, P. F. 1975. *Isis* 66:504–33
116. Rhoads, D. C., Lutz, R. A., Revelas, E. C., Cerrato, R. M. 1981. *Science* 214: 911–12
117. Richard, J. 1907. *L'Oceanographie*. Paris: Vuibert & Nony
118. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolteon, E. T., Britton, R. J. 1963. *Carnegie Inst. Wash. Publ. No. 607*. 521 pp.
119. Rona, P., Bostrom, K., Laubier, L., Smith, K. L., eds. 1984. *Hydrothermal Processes at Sea Floor Spreading Centers*, pp. 677–709. New York: Plenum. 796 pp.
120. Rowe, G. T., Sibuet, M., 1983. In *Deep-Sea Biology*, ed. G. T. Rowe, pp. 81–95. New York: Wiley
121. Ruby, E. G., Jannasch, H. W. 1982. *J. Bacteriol.* 149:161–65
122. Ruby, E. G., Wirsen, C. O., Jannasch, H. W. 1981. *Appl. Environ. Microbiol.* 42:317–24
123. Sanders, H. L., Hessler, R. R., Hampson, G. R. 1972. *Deep-Sea Res.* 12:845–67
124. Saunders, P. M., Fofonoff, N. P. 1976. *Deep-Sea Res.* 23:109–11
125. Schmidt-Lorenz, W. 1967. *Bull. I.I.R.*, 2 and 4:1–59
126. Schwarz, J. R., Landau, J. V. 1972. *J. Bacteriol.* 109:945–48
127. Schwarz, J. R., Landau, J. V. 1972. *J. Bacteriol.* 112:1222–27
128. Deleted in proof
129. Seki, H., Wada, E., Koite, I., Hattori, A. 1974. *Mar. Biol.* 26:1–4
130. Shilo, M. 1979. *Strategies of Microbial Life in Extreme Environments*. Life Sci. Rep. 13, Weinheim/New York: Verlag Chemie
131. Silver, M. W., Alldredge, A. K. 1981. *J. Mar. Res.* 39:501–30
132. Silver, M. W., Shanks, A. L., Trent, J. D. 1978. *Science* 201:371–73
133. Simoneit, B. R. T., Lonsdale, P. F. 1982. *Nature* 295:198–202
134. Sleigh, M. A., Macdonald, A. G., eds. 1972. *The Effects of Pressure on Organisms*. Symp. Soc. Exp. Biol., 26:516. New York: Academic
135. Smith, W. P., Pope, D. H., Landau, J. V. 1975. *J. Bacteriol.* 124:582–84
136. Somero, G. N. 1978. In *Biochemical and Biophysical Perspectives in Marine Biology*, ed. D. C. Malins, J. R. Sargent, 4:1–27. London: Academic
137. Somero, G. N., Siebenaller, J. F., Hochachka, P. W. 1983. In *The Sea*, ed. G. T. Rowe, 8:261–330. New York: Wiley
138. Southward, E. C. 1982. *J. Mar. Biol. Assoc. UK* 62:889–906
139. Southward, A. J., Southward, E. C., Dando, P. R., Rau, G. H., Felbeck, H., et al. 1981. *Nature* 293:616–20
140. Stahl, D. A., Lane, D. J., Olsen, G. J., Pace, N. R. 1984. *Science* 224:409–11
141. Stetter, O. 1982. *Nature* 300:258–60
142. Suess, E. 1980. *Nature* 288:260–63
143. Suzuki, I. 1974. *Ann. Rev. Microbiol.* 28:85–101
144. Tabor, P. S., Colwell, R. R. 1976. *Proc. MTS/IEEE OCEANS 1976*, pp. 13D1–13D4, Washington DC: Marine Tech. Soc.
145. Tabor, P. S., Deming, J. W., Ohwada, K., Davis, H., Waxman, M., et al. 1981. *Microb. Ecol.* 7:51–65
146. Taylor, C. D. 1978. *Arch. Biochem. Biophys.* 191:375–84
147. Taylor, C. D. 1979. *Appl. Environ. Microbiol.* 37:42–49
148. Taylor, C. D. 1979. *Undersea Biomed. Res.* 6:147–54
149. Taylor, C. D., Jannasch, H. W. 1976. *Appl. Environ. Microbiol.* 32:355–59
150. Terwilliger, R. C., Terwilliger, N. B., Arp. A. 1983. *Science* 219:981–83
151. Thom, S. R., Marquis, R. E. 1981. In *Underwater Physiology*, ed. A. J. Bachrach, M. M. Matzen, 7:667–73. Bethesda, MD: Undersea Med. Soc.
152. Thom, S. R., Marquis, R. E. 1984. *Appl. Environ. Microbiol.* 47:780–87
153. Turekian, K. K., Cochran, J. K. 1981. *Science* 214:909–10
154. Turner, J. T. 1977. *Mar. Biol.* 40:249–59
155. Turner, J. T. 1979. *Trans. Am. Microsc. Soc.* 98:131–35
156. Tuttle, J. H., Wirsen, C. O., Jannasch, H. W. 1983. *Mar. Biol.* 73:293–99
157. Vinogradov, M. E., Tseitlin, V. B.

1983. In *Deep-Sea Biology*, ed. G. T. Rowe, pp. 123–65. New York: Wiley
158. Wada, E., Koike, I., Hattori, A. 1975. *Mar. Biol.* 29:119–24
159. Watson, S. W., Hobbie, J. E. 1979. In *Native Aquatic Bacteria: Enumeration, Activity, and Ecology*, ed. J. W. Costerton, R. R. Colwell, pp. 82–88. Philadelphia: Am. Soc. for Testing and Materials
160. Weiner, R. M., Devine, R. A., Powell, D. M., Dagasan, L., Moore, R. L. 1983. *Int. J. Syst. Bacteriol.* In press
161. Welhan, J. A., Craig, H. 1979. *Geophys. Res. Lett.* 6:829
162. Williams, A. J. III, Fairhurst, K. 1977. *Ocean Engineer.* 4:205–10
163. Williams, D. L., von Herzen, R. P., Sclater, J. G., Anderson, R. N. 1974. *Geophys. J. R. Astron. Soc.* 38:587–608
164. Wirsen, C. O., Jannasch, H. W. 1975. *Mar. Biol.* 31:201–8
165. Wirsen, C. O., Jannasch, H. W. 1976. *Environ. Sci. Technol.* 10:880–87
166. Wirsen, C. O., Jannasch, H. W. 1983. *Mar. Biol.* 78:69–73
167. Wittenberg, J. B., Morris, R. J., Gibson, Q. H., Jones, M. L. 1981. *Science* 213:244–46
168. Yayanos, A. A., Dietz, A. S. 1982. *Appl. Environ. Microbiol.* 43:1481–89
169. Yayanos, A. A., Dietz, A. S. 1983. *Science* 220:497–98
170. Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1979. *Science* 205:808–10
171. Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1981. *Proc. Natl. Acad. Sci. USA* 78:5212–15
172. Yayanos, A. A., Dietz, A. S., Van Boxtel, R. 1982. *Appl. Environ. Microbiol.* 44:1356–61
173. Zillig, W., Schnabel, R., Tu, J., Stetter, K. O. 1982. *Naturwissenschaften* 69:197–204
174. Zimmerman, A. M., ed. 1970. *High Pressure Effects on Cellular Processes.* New York: Academic. 324 pp.
175. ZoBell, C. E. 1946. *Marine Microbiology.* Waltham, MA: Chronica Botanica Co.
176. ZoBell, C. E. 1968. *Bull. Misaki Mar. Biol. Inst.*, Kyoto Univ. 12:77–96
177. ZoBell, C. E. 1970. See Ref. 174, pp. 85–130
178. ZoBell, C. E., Kim, J. 1972. In *The Effects of Pressure on Animals*, ed. M. A. Sleigh, A. G. Macdonald, pp. 125–46. Cambridge Univ. Press
179. ZoBell, C. E., Johnson, F. H. 1949. *J. Bacteriol.* 57:179–89

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BIOLOGY OF IRON- AND MANGANESE-DEPOSITING BACTERIA

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INTRODUCTION

Bacteria have long been recognized for their ability to deposit iron hydroxide or manganese oxide in structures outside their cells (15, 92, 190). Indeed, the classical Fe- and Mn-depositing bacteria, *Gallionella, Sphaerotilus, Leptothrix,* and *Clonothrix,* were all described during the nineteenth century (25, 143). Commonly known as "iron bacteria," these and many other Fe- and Mn-depositing organisms occur in ocherous deposits and ferromanganese deposits that form in neutral waters of lakes, ponds, swamps, bogs, drainage ditches, and chalybeate springs. Fe- and Mn-depositing bacteria also occur in wells and water-distribution systems, where they can cause significant clogging problems.

Some of these bacteria are recognized in natural environments by the morphology of their metal-encrusted structures. Some have also been isolated, but few have been studied extensively in axenic cultures. For those studied in detail, most of the information available is structural. In some cases enzymatic mechanisms of Fe and Mn deposition have been shown. Proposals for chemoautotrophic and mixotrophic growth have been made; however, with the possible exception of chemoautotrophy in *Gallionella,* most of the proposals are unsubstantiated by firm experimental evidence. In general, physiological and genetic aspects of Fe and Mn deposition by these bacteria are poorly understood.

Recent interest in the economic, environmental, and geochemical importance of metal-transforming microorganisms (19, 55, 97, 119, 171) has rekindled interest in the biology of Fe- and Mn-depositing bacteria. This renewed interest arises, in part, from the realization that some of these bacteria may be involved in the formation of ferromanganese deposits containing economically important trace metals. In addition, it is suggested that Fe- and Mn-depositing bacteria may be useful for recovering valuable and toxic metals from dilute aqueous milieux.

The purpose of this article is to review the literature on the biology of Fe- and Mn-depositing bacteria, with the intent of identifying and, if possible, clarifying past and present problems. The article is meant to guide students and researchers who are interested in the occurrence and activity of these bacteria in natural environments. It should also guide those interested in their isolation, enrichment, cultivation, and taxonomy. Recent work on structural, physiological, and genetic aspects of Fe and Mn deposition by these bacteria is also reviewed.

In recent years, Fe-oxidizing thiobacilli (33, 175) and other acidophilic Fe-oxidizing chemolithotrophs have been deemed "true iron bacteria" (113, 135, 194) in the sense originally intended by Winogradsky (190). The biology of the Fe-oxidizing thiobacilli has been adequately reviewed recently (see 55, 113, 135a, and references therein); therefore, except for mentioning some recent work on the genetics of *Thiobacillus ferrooxidans,* acidophilic Fe oxidizers will not be included in this article.

PAST PROBLEMS

Numerous articles on various aspects of the biology of Fe- and Mn-depositing bacteria have been published since Winogradsky first coined the term "iron bacteria" (190). During his studies, Winogradsky observed that the filamentous bacterium, *Leptothrix ochracea,* thrived in Fe-containing enrichment cultures without the addition of organic nutrients. By analogy with his previous studies on sulfur bacteria, he postulated but never proved (143) that *L. ochracea* could derive energy from the oxidation of Fe to fix CO_2 and grow autotrophically.

In spite of excellent descriptive work by several subsequent workers (e.g. 32, 84), for the next 60 years the biology of iron bacteria remained problematic. During the 1940s Pringsheim (143) critically reviewed "a vast literature of unequal value" covering nearly all of the previous work on these bacteria. Pringsheim noted "a peculiar hesitation and uncertainty regarding autotrophy in iron bacteria" and a clear need for "closely watched culture experiments" combined with "careful observations in nature" during investigations of these bacteria. Pringsheim also attempted to clarify the taxonomy of iron bacteria, but in doing so he created some new problems (see below). Nevertheless, his thorough review of the historical literature is a valuable reference and recommended to those interested in the biology of these bacteria.

Another valuable reference of the same era is the review of Fe-transforming bacteria in water distribution systems by Starkey (166). This article, still used as a primary reference for water system workers (165), contains lucid discussion of chemical and physiological aspects of bacterial Fe transformations, including Fe deposition by nonspecific heterotrophic bacteria as well as by the classical iron bacteria. Acknowledging some severe difficulties with cultivating iron bacteria, Starkey pointed out that the study of these bacteria may be "limited almost entirely to microscopic observations and simple chemical tests" (166). As indicated below, many of the difficulties with culturing these bacteria remain unresolved.

After 1950, the opportunity existed for modern experimental work on Fe- and Mn-depositing bacteria, but some problems persisted. During the 1950s Wolfe and coworkers reported phase contrast and electron microscopic (EM) observations on *Gallionella, Crenothrix,* and *Clonothrix* in crude samples and in cultures (186, 191–193). In addition, Kucera & Wolfe's (107) widely used FeS-enrichment medium for *Gallionella* was described. Unfortunately, Wolfe did not pursue his studies of Fe- and Mn-depositing bacteria further. He found full-time study of these organisms "too difficult to be profitable" (194).

Meanwhile, other researchers, particularly Mulder and his coworkers (127, 131) in Holland and Stokes (168) in the United States, had begun investigations on Fe- and Mn-depositing sheathed bacteria of the *Sphaerotilus-Leptothrix* group (40, 185); but immediately problems arose concerning nomenclature.

The crux of the nomenclature problems was Pringsheim's previous demonstration (149) that forms of the sheathed iron bacteria could change under different cultural conditions. Because of his observations, Pringsheim (143, 144) argued that most forms previously designated as various species of *Leptothrix, Cladothrix,* and *Clonothrix* should be included in the genus *Sphaerotilus* along with *S. natans*. He proposed abolishment of the generic names *Leptothrix, Cladothrix,* and *Clonothrix* and recognized instead only two species: *S. natans* and *S. discophorus*. *S. discophorus* was distinguished by its ability to deposit ferromanganese oxide in the sheath. *S. natans* deposited primarily iron hydroxide.

Pringsheim's attempt to clarify the taxonomy of the sheathed iron bacteria created considerable controversy. For example, while Stokes (168), Wolfe (191), and several others adopted Pringsheim's proposed changes, Mulder (127, 131) and still others (141) did not accept his abolishment of the genus *Leptothrix*. Mulder (131) correctly pointed out that Pringsheim had never isolated any bacteria from his enrichment cultures that contained *Leptothrix ochracea* sheaths. Therefore, he had not demonstrated the true identity of the organisms that produced the sheaths. Nonetheless, Pringsheim reasoned—albeit erroneously—that, because the empty sheaths in his cultures resembled Fe-encrusted *S. natans* sheaths, *L. ochracea* must be a form of *S. natans*.

Subsequent work in Mulder's laboratory (127) demonstrated that *S. natans* and *L. ochracea* are different organisms. In low-nutrient, Fe-containing enrichment cultures, *L. ochracea* synthesized and vacated iron-encrusted sheaths very rapidly, leaving behind large masses of empty sheaths. *S. natans* preferred higher concentrations of nutrients for growth and did not vacate its sheaths. Citing this evidence and giving several practical reasons, Mulder and coworkers (130, 185) rejected Pringsheim's classification in favor of one in which the genera *Sphaerotilus* and *Leptothrix* were maintained. According to Mulder & Dienema (130), *L. ochracea,* which may account for most of the Fe-encrusted sheaths observed in natural iron springs, has yet to be isolated in axenic cultures.

On the other hand, several other *Leptothrix* species have been isolated and studied in axenic cultures. Depending on whether or not Pringsheim's nomenclature was followed, the isolates have been classified as either *Sphaerotilus discophorus* or as a *Leptothrix* spp. Generally, between 1950 and 1975 authors from the US followed Pringsheim's nomenclature, whereas European and Soviet authors did not. After the publication of the eighth edition of

Bergey's Manual (1974), Mulder's description of the genus *Leptothrix* (129) gained acceptance. Unfortunately, the acceptance has not been universal (123, 149, 150). Thus the past inconsistencies of nomenclature still can cause problems to those unfamiliar with their history.

OCCURRENCE AND ACTIVITY IN NATURE

Fe- and Mn-depositing bacteria are ubiquitous. They have been detected in samples from almost every compartment of the biosphere where iron hydroxide and ferromanganese oxide deposits are found, ranging from deep-sea hydrothermal vent regions (59, 93) to the surface of desert rocks (41, 106, 173). Partial listings of specific microorganisms that are known to deposit Fe and Mn oxides in cultures or in natural environments have been published (36, 55, 74, 103, 108, 109, 113, 119, 135, 145). These lists include the classical iron hydroxide– and ferromanganese-depositing bacteria, *Sphaerotilus, Gallionella, Leptothrix, Crenothrix,* and *Clonothrix,* the more recently described *Metallogenium* and *Siderocapsaceae,* and the ferromanganese-depositing hyphal budding bacteria, *Pedomicrobium* and *Hyphomicrobium*.

Such lists also include various heterotrophic bacteria (Table 1, page 526), fungi (55, 119), algae, and protozoa (119, 145), all of which have been shown to deposit Mn and Fe oxides under laboratory conditions or in natural environments. Such lists should also include the *Blastocaulis-Planctomyces* group, which have recently been shown to deposit Mn in their stalks (157), and magnetotactic bacteria (18), which accumulate significant amounts of magnetite (Fe_3O_4) inside their cells. The widespread occurrence of Fe- and Mn-depositing microorganisms suggests that they may be important in the formation of natural accumulations of ferromanganese oxides that occur frequently as coatings, concretions, flocculent particles, and surface films in soils, sediments, and watery environments.

In the past, biological responsibility for Fe and Mn deposition in chalybeate springs, wells, and water-distribution systems has been taken as self-evident. Indeed, bacteria such as *Gallionella* and *Leptothrix ochracea* deposit copious amounts of their iron hydroxide–encrusted stalks and sheaths in the environments where they grow (36, 82, 84, 131, 166, 191). Others such as *Crenothrix* (192) and *Clonothrix* (193) frequently occur in large masses of morphologically distinct filaments that may become encrusted with Fe or Mn oxide.

The involvement of Fe- and Mn-depositing bacteria in the formation of ferromanganese ore deposits in lakes has also been taken as self-evident by Soviet microbiologists, who cite reports of *Leptothrix* spp. and other Fe- and Mn-depositing bacteria including *Metallogenium* in sedimentary environments where lake ores form (108, 109). However, geochemists and microbiologists

outside the Soviet Union have not been in total agreement concerning the contribution of microorganisms to the formation of ferromanganese concretions (28, 29, 37, 51, 85, 101, 119).

Generally, geochemists have agreed that microorganisms, especially bacteria and algae (37), may create microenvironmental conditions (e.g. elevated pH and Eh) conducive to oxidation of Fe and Mn. They also recognize that microorganisms may promote enzymatic catalysis of oxidation reactions (28, 85). However, some geochemists have had difficulties justifying a biological mechanism for ferromanganese-concretion formation because they have found it "difficult to assess the role of bacteria for systems (iron and manganese) where a purely physicochemical mechanism applies" (28).

This problem has been difficult to resolve because direct evidence for bacterial participation (e.g. sterile control experiments) has been lacking in the microbiological literature. In fact, some geochemists (29) perceived the proper control experiments as impossible to conduct in sedimentary environments, arguing that, in addition to Fe- and Mn-depositing bacteria, other microbial populations (e.g. sulfate-reducers) may be required to provide the reducing conditions that insure a steady supply of Fe and Mn to the surface where the concretions form.

Such pessimism has not been shared by microbiologists aware of the assortment of powerful microscopic techniques, specific metabolic inhibitors, and other tools available for probing microbial presence and activity in microhabitats (154). During the last decade, since the first EM observations of bacterial forms in marine iron ore (146) and on manganese nodules (110), an increasing number of reports have appeared in the literature in which scanning (SEM) or transmission (TEM) electron microscopy have been employed to examine Fe- and Mn-encrusted microorganisms directly in natural samples.

Such work has clearly demonstrated intimate associations of microorganisms with Fe and Mn minerals in fresh- and saltwater ferromanganese concretions (26, 65, 71, 72, 174), bog ore and iron spring deposits (27, 35, 133), rock varnish (41, 106, 173), detrital particles in lakes and ponds (76, 87, 94, 102, 157, 158), water-distribution systems (120, 147, 148, 179–183), fjords (59, 174), and surface films of swamps and shallow ponds (61, 68, 71; see also Figures 1 and 2). Similar to the previous light microscopic evidence showing abundant Fe-encrusted bacterial structures in iron spring deposits, the recent EM evidence strongly suggests—but does not prove—that microorganisms are involved in the formation of the mineral deposits.

Recognizing the deficiencies of relying on only microscopic techniques, Emerson & Nealson and their coworkers (59, 135b, 174) employed ^{54}Mn tracer methods in conjunction with metabolic poisons and EM observations to study environmental oxidation rates of Mn^{2+} at the H_2S/O_2 interface in Saanich Inlet

on the west coast of Canada and in a Norwegian fjord. These studies showed that metal-encrusted bacteria were the predominant microorganisms present in particles at the Saanich Inlet interface, and that bacterial catalysis was involved in Mn^{2+} oxidation in both fjords. Other combined microbiological and geochemical techniques have been developed recently by Nealson and coworkers. In one such technique metabolic poisons are used in dialysis probes or "peepers." Poisoned and nonpoisoned peepers have been used to study kinetics of microbial Mn oxidation in sediments (98).

More traditional approaches, including enumeration of Mn-depositing bacteria and measurement of geochemical and limnological parameters, have also been employed recently to demonstrate bacterial involvement in Mn oxidation in Oneida Lake, NY (31) and in a swamp in Ithaca, NY (67, 68). The latter studies demonstrated that numbers of viable *Leptothrix* in the swamp surface correlated directly with particulate Fe and Mn in the surface water, indicating these sheathed bacteria were responsible for the deposition of ferromanganese in the film (67).



Figure 1 TEM thin section showing a *Leptothrix* sp. filament in a sample from a ferromanganese film of a swamp in Ithaca, NY (see 67–68). The section passed through the flat holdfast end of the filament, revealing two cells within the sheath tube. Note protuberances of the cell envelope, which contact the fibrilar electron-dense sheath material. Bar = 0.5 μm.

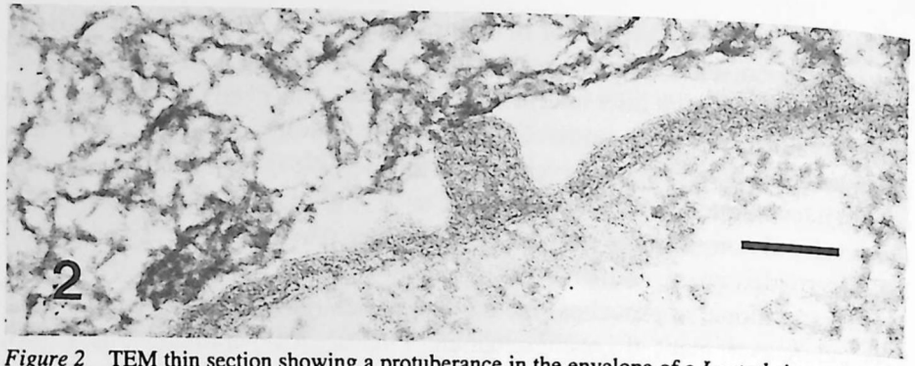


Figure 2 TEM thin section showing a protuberance in the envelope of a *Leptothrix* sp. cell in a sample from a ferromanganese film of a pond near Kiel, W. Germany (see 73). In this instance, the glutaraldehyde-fixed samples were extracted with acidified leukoberbelin blue to remove Mn oxide (70). Ruthenium red (RR) was then applied to stain acidic extracellular polymers. Note intimate contact between RR-stained polymers and the outer membrane of the cell envelope on the protuberance. Bar = 0.1 μm.

ENRICHMENT AND ISOLATION

Crenothrix, Clonothrix, Leptothrix ochracea, *and* Gallionella

Despite their common occurrence in natural samples, difficulties are frequently encountered in cultivating many Fe- and Mn-depositing bacteria. *Crenothrix polyspora, Clonothrix* sp., and *Leptothrix ochracea,* for example, have never been obtained in axenic cultures in spite of dedicated efforts to isolate them (130, 131, 187, 191–194). Isolation of *Gallionella* has been reported by Hanert (80, 82) and Nunley & Kreig (138); however, the validity of Hanert's bouillon-yeast extract broth test for contaminants was questioned by Balashova (10), and Nunley & Kreig's formalin-resistance method could not be reproduced by Hanert (82). Because all four of the bacterial species are found in similar habitats (82, 166, 191–193), difficulties encountered in cultivating them could stem from similar nutritional requirements. Requirements for O_2, Fe^{2+}, and CO_2 and an intolerance for organic nutrients have been shown for *Gallionella* (82), but nutritional requirements of the other three bacterial species are unknown.

Recently, some clues on *Crenothrix polyspora* nutrition were found during an ultrastructural investigation of this interesting sheathed bacterium (187). In this investigation, *C. polyspora* growing in well water were found to contain extensive stacked internal membrane systems reminiscent of those in some photosynthetic, nitrifying, or methane-oxidizing bacteria. Because no photosynthetic pigments were detected and the cultures were unable to nitrify, the authors concluded that *C. polyspora* may be able to oxidize methane (187). Further, it was determined that methane was present in the well water, but no

investigations of the possible methane oxidation were reported. Very little Fe and only traces of Mn were detected at the bases of the *C. polyspora* filaments, so it seems unlikely that these bacteria were oxidizing extensive amounts of the metals for energy. Thus, the possibility that *C. polyspora* may oxidize methane or other single-carbon compounds as energy sources remains open.

It is possible that these difficult-to-isolate Fe- and Mn-depositing bacteria require other organisms to create conditions conducive for their growth. The possibility of synergistic associations of *Gallionella* with other bacteria has been suggested (8, 138), and several synergistic combinations of Mn-depositing bacteria with other bacteria, fungi, and algae have been reported (see references in 55, 119). Furthermore, it can be argued that gradient-loving bacteria such as *Gallionella* are likely to depend on sulfate-reducing and other anaerobic bacteria to maintain microaerophilic conditions in their environments.

Other Leptothrix *spp. and* Sphaerotilus natans

Isolation and cultivation of most other Fe- and Mn-depositing bacteria have presented fewer problems. For example, most *Leptothrix* spp. and *Sphaerotilus natans* can be easily isolated by employing Winogradsky's (190) classical plant extract–enrichment procedures or numerous other procedures including continuous-flow enrichments and a variety of media formulations for isolation (40, 130, 141, 185).

Often it is preferable to isolate *Leptothrix* spp. without enrichment, because enrichment cultures may lead to isolations of only one of several *Leptothrix* species encountered in nature (130). This is easily achieved by plating natural material directly onto Mn-containing agar media. Some freshly isolated *Leptothrix* spp. grow poorly on elevated concentrations of organic nutrients (130; W. C. Ghiorse, unpublished observations); therefore, as a rule *Leptothrix*-isolation media are low in organic nutrients (131, 155, 156).

Leptothrix spp. and other Fe- and Mn-depositing bacteria are routinely isolated and enumerated in my laboratory on media consisting of natural water to which 10 mg of $MnSO_4 \cdot H_2O$ plus 15 g of agar liter^{-1} are added before autoclaving. Such media have been employed to isolate *Leptothrix discophora* (Figures 3 and 4) and other Mn-depositing bacteria from several different aquatic environments (67, 68, 72, 189).

Siderocapsaceae

Enrichment procedures for bacteria of the *Siderocapsaceae* group have been described by Hanert (83). In these procedures small amounts of yeast extract (0.007%) and $FeCO_3$ or $MnCO_3$ are added to 20 or 40 liters of natural water in aquaria. After a succession of *Leptothrix* and sulfate-reducing bacteria, masses of ocherous iron develop on water surface that contain bacteria typical of the

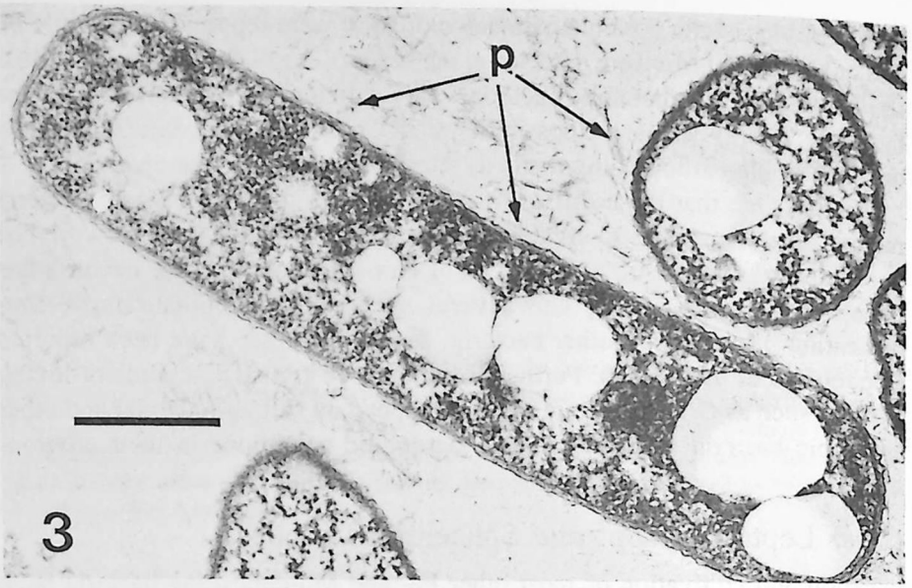


Figure 3 EM thin section showing *Leptothrix discophora* SS-1 cells grown in PYGV medium (69) with no Mn added. Note abundant electron-dense extracellular material (*p*), which appears to be unorganized sheath polymers. The large transparent bodies inside cells contain PHB. Bar = 0.5 μm.

Siderocapsaceae group. Subsequent isolation of the *Siderocapsaceae* bacteria from the ocherous masses is difficult, however, because they tend to lose their identifying morphological features when they form colonies on agar (83). Obviously, more work is needed to develop methods for reliable isolation of the members of this group that are thought to be major contributors to iron and manganese deposition in lakes (108).

Hyphal Budding Bacteria

Fe- and Mn-depositing *Pedomicrobium* spp. and other metal-depositing hyphal budding bacteria are relatively easily enriched and isolated from natural samples by employing modified procedures developed for isolating hyphal budding bacteria (69, 71). *Pedomicrobium* sp. were first isolated from podzolic soil by Aristovskaya (6) and have been reisolated since by Gebers & Hirsch (63, 64) employing Aristovskaya's original humic gel medium as well as other media. Like most of the isolation procedures used for budding bacteria (89), prolonged incubation (3–9 months) and addition of vitamins to the isolation medium is recommended for *Pedomicrobium* spp. (64).

Other Fe- and Mn-depositing hyphal budding bacteria have been isolated from such various aquatic and terrestrial environments as coastal seawater (88), hydroelectric pipelines and lakes (120, 180–183), brackish-water ferromanganese concretions (71), drinking-water systems and thermal springs (58, 161),

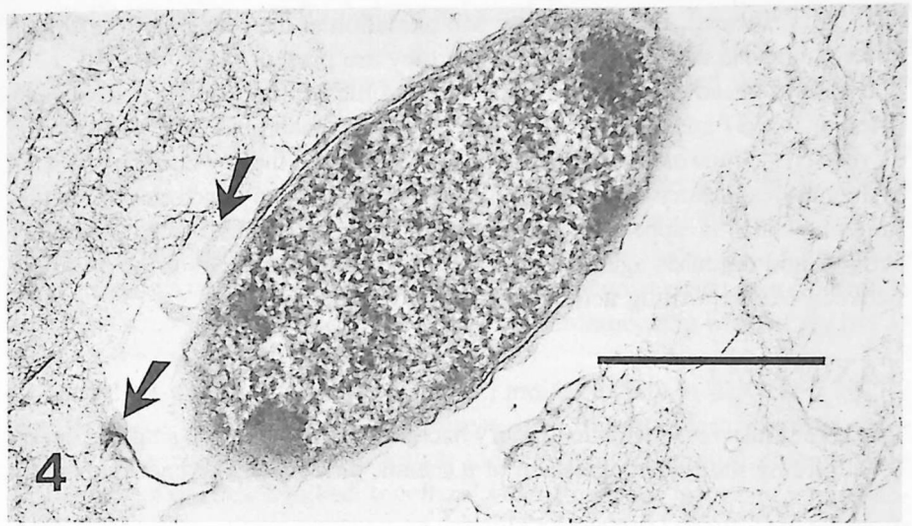


Figure 4 TEM thin section showing a portion of a *Leptothrix discophora* SS-1 cell grown in PYGV medium (69) with Mn added. *Arrows* indicate extensions of the outer membrane at points where they contact the electron-dense matrix of Mn oxide surrounding the cell. Bar = 0.5 μm.

wetlands (68, 69), and soil (128, 184). Generally, long-term enrichments employing low-nutrient, low-phosphate media or media consisting of natural water or soil extract with Fe or Mn added at low concentrations have been successful.

Other Heterotrophs

A variety of heterotrophic bacteria from diverse sources has been shown to deposit Mn oxide in axenic cultures; however, relatively little interest has been shown in Fe deposition by such bacteria. Because nonspecific Fe-depositing ability has been demonstrated in many heterotrophs (114, 115), it is likely that any bacteria that deposit Mn oxides will also deposit iron by hydroxides if provided with the proper cultural conditions. The Mn-depositing bacteria listed in Table 1 reveal themselves by depositing brown Mn oxide in or near their colonies on Mn-containing agar media. In most cases, the media employed contained low concentrations of organic nutrients and of Mn^{2+} (17, 20, 21, 76, 134, 159, 160, 172, 184), but in a few instances media with high concentrations of organic nutrients (~0.5%) and Mn (~0.1%) were used (43, 56, 111, 195).

Ehrlich (48, 49, 52, 54) has isolated several Mn-oxidizing heterotrophic bacteria, including *Arthrobacter* sp., *Oceanospirillum* sp., and *Vibrio* sp., from deep-sea ferromanganese nodules and sediments by employing standard microbiological media (e.g. Difco nutrient broth). In such rich media, the Mn-oxidizing bacteria do not deposit Mn oxides in their colonies or around

their cells. Instead, they accelerate Mn oxidation at the surface of preformed ferromanganese oxides or MnO_2 when they are present (4, 5, 49, 50).

It should be noted in passing that most of the Mn-depositing heterotrophs listed in Table 1 are aerobes. A few are facultative anaerobes (e.g. *Aeromonas, Citrobacter, Enterobacter, Proteus,* and *Vibrio*), but these would be expected to contain respiratory systems. As discussed below, many bacterial Mn oxidation mechanisms appear to be physiologically associated with respiratory activity and defenses against O_2 toxicity. Thus there appears to be a linkage between Mn-depositing activity and oxidative metabolism.

TAXONOMY

It is sometimes reasonable to classify bacteria on the basis of a single property (e.g. gliding motility, possession of a sheath, photosynthetic energy metabolism). However, groups formed around single properties usually are found to be heterogenous with respect to other properties. This presents significant problems for taxonomists interested in a classification based on natural relationships (139).

Table 1 Bacteria known to deposit Mn oxide in axenic cultures

Part of *Bergey's Manual* (25)		Genus	References
2	Gliding bacteria	*Cytophaga*	76, 134
3	Sheathed bacteria	*Leptothrix*	129; Figure 4
4	Budding and appendaged bacteria	*Hyphomicrobium*[a]	71, 88, 128, 182; Figure 6
		Pedomicrobium	7, 63
		Metallogenium[b]	198; Figure 8
		Caulobacter	76
6	Spiral and curved bacteria	*Oceanospirillum*	52
7	Gram-negative aerobic rods	*Pseudomonas*	24, 42, 76, 134, 160, 172, 196
8	Gram-negative facultatively anaerobic rods	*Enterobacter, Proteus*	24
		Citrobacter	42
		Vibrio	52
		Aeromonas	134, 159
	Uncertain affiliation	*Flavobacterium*	134
12	Gram-negative, chemolithotrophic bacteria	*Siderocapsaceae*	83
15	Endospore-forming rods	*Bacillus*	17, 24, 137, 184; Figure 5
17	Actinomycetes and related organisms	*Arthrobacter*	24, 48, 172, 184, 199
		Nocardia	176
		Streptomyces	22, 111, 176

[a] *Hyphomicrobium* spp. isolated on peptone-containing media may belong in *Hyphomomas* or *Pedomicrobium* (124).

[b] According to Zavarzin (197, 198) only the type species, *Metallogenium personatum,* has been obtained in axenic cultres, but these apparently have not survived. A second species, *M. symbioticum,* has been obtained only in cocultures with various fungi and *Bacillus* spp. As discussed in the text, *Metallogenium*-like forms are also commonly observed in samples from natural environments, but their biological origin is uncertain.

A cursory glance at the list of Mn-depositing bacteria (Table 1) reveals that this property is found in many different morphological and physiological groups in *Bergey's Manual* (25). As suggested in the previous section, most Mn-depositing bacteria can probably also deposit iron hydroxides nonspecifically. Strict anaerobes would not be expected to deposit either iron hydroxide or Mn oxide because the redox potential of environments in which they live is too low for the oxidation to occur.

The diversity of bacteria listed in Table 1 indicates that Mn-depositing ability may have evolved in many unrelated organisms. Because there appears to be a linkage between oxidative metabolism and Mn-oxidizing ability, it is possible that both properties evolved together. One advantage of possessing Mn-oxidizing activity would be that energy could be derived from the oxidation. It is possible that some of these bacteria may have evolved this ability (57, 96a, 113, 119); but as discussed below, no unequivocal evidence for this exists. Other possible selective advantages of metal deposition, including nutrient scavenging and detoxification, have been proposed (77), but such properties cannot be used to classify them.

A detailed discussion of the taxonomy of Fe- and Mn-depositing bacteria is beyond the scope of this review because it would involve discussion of the many groups that contain them. However, it should be recognized that many Fe- and Mn-depositing bacteria form extracellular structures, which have been used to classify them. In particular, one group in *Bergey's Manual* (25), the *Siderocapsaceae,* are identified primarily by their metal-impregnated extracellular mucoid material. Whether the *Siderocapsaceae* form a valid taxonomic unit has been questioned recently by Dubinina & Zhdanov (46), who showed that some *Siderocapsa* spp. appear to be *Arthrobacter* spp. In addition, Schmidt (158) recently isolated *Vibrio* spp. or *Pseudomonas* spp. from his cultures of *Siderocapsa geminata.* (For further details, see discussion of *Siderocapsaceae* capsule and cell-wall structure below.) These findings indicate that the taxonomy of this group is in disarray and requires further study.

STRUCTURES ASSOCIATED WITH FE AND MN DEPOSITION

Intracellular Structures

Most Fe- and Mn-depositing bacteria that have been examined ultrastructurally contain the usual array of prokaryotic structures, including a nucleoid region, ribosomes, storage inclusions (PHB and glycogen are common), plasma mem-

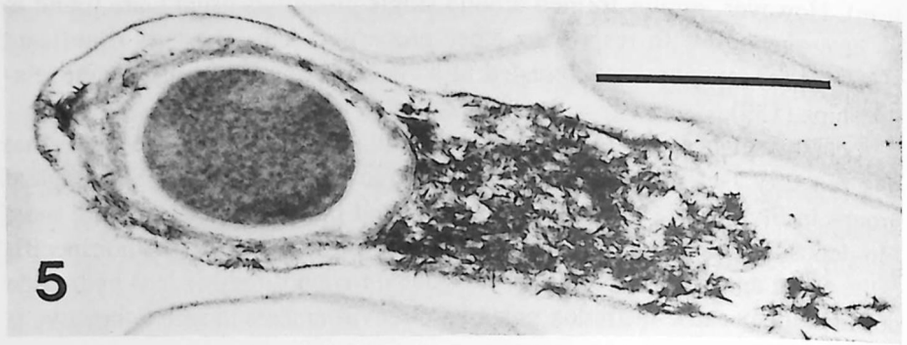


Figure 5 TEM thin section showing Mn oxide deposited inside the sporangial wall, but outside the spore coat of *Bacillus sphaericus*, after one month of incubation on a Mn-containing agar medium. Bar = 0.5 μm.

brane, and flagella. Both gram-positive and gram-negative cell envelopes have been found. Except for intracellular structures containing magnetite (magnetosomes) in magnetotactic bacteria (12) and a report of electron-dense spherical bodies in the cytoplasm of *Gallionella* (11), no morphologically recognizable intracellular structures containing Fe or Mn have been reported in these bacteria. As a rule, metal-oxide deposits are found outside the cell envelope.

Bacillus spp. seem to be exceptions to this generalization, because they accumulate metal oxides inside their sporangial cell walls after sporulation (Figure 5). Apparently, Mn-depositing activity is a property of many *Bacillus* spp., which form metal oxides in association with spores (100, 153, 184; W. C. Ghiorse, unpublished observations). Other apparent exceptions are intracellular Mn-oxide deposits reported in the cultures of Nealson & Tebo (136). In my experience, such internal Mn-oxide deposits frequently are observed inside disorganized cells that presumably were not viable at the time of fixation.

These observations illuminate an important point concerning possible influences of bacteria on sedimentary metal deposition. They indicate the possibility for formation of bacterial microfossils—metal-encrusted bacterial corpses—that could contribute to metal deposition in sedimentary environments (16). In addition to the microfossils described in precambrian rocks (32a), fossils resembling bacterial cells have been documented by EM in a variety of sedimentary samples (27, 38, 39, 65), indicating that bacterial membranes and wall polymers can contribute significantly to metal deposition in sediments.

Unorganized Extracellular Polymers

Bacteria produce a variety of extracellular polymers that may or may not form distinct structures outside the cell wall. In general, Fe- and Mn-depositing bacteria that do not form well-organized and recognizable extracellular structures have attracted little attention from observers of natural samples. Thus they remain poorly characterized.

Most of the nondescript Fe- and Mn-depositing bacteria, like other bacteria in natural environments (34), would be expected to produce acidic exopolymers (slime layers) even if they do not form distinctive structures. Although these bacteria remain poorly characterized, ultrastructural evidence showing bacteria enmeshed in metal-impregnated polymer matrices on ferromanganese concretions has been obtained recently (65). However, isolation of these metal-depositing slime-forming bacteria from enrichment cultures has not been totally successful. Fortunately, some of the bacteria in the matrix of Baltic Sea concretions could be recognized as hyphal budding bacteria that subsequently were isolated from enrichment cultures (71). The isolates were similar to other strains of budding bacteria studied previously (69, 70, 120) in that they produced ruthenium red (RR)-stainable acidic extracellular polymers upon which Fe and Mn oxides were deposited.

The association of acidic extracellular polymers with Fe and Mn oxides in 12 strains of budding bacteria was investigated by employing cultural tests for Fe and Mn deposition and using EM to demonstrate RR-stainable polymers (Table 2). In this study only four strains (strains 868, 869, E-211, and B-4) deposited Mn and Fe, whereas seven deposited only Fe and one (W54) deposited neither metal. Furthermore, RR-stainable polymers were detected on all strains except W54. These results further indicate the association of acidic

Table 2 Fe- and Mn-oxide deposition and ruthenium-red (RR) staining of hyphal budding bacteria[a]

Genus	Strain	Mn[b]	Fe[b]	RR[c]
Pedomicrobium sp.	868	+	+	+
Pedomicrobium sp.	869	+	+	+
P. magnicum	E-211	+	+	+
P. ferrugineum	S-122	–	+	+
Hyphomicrobium sp.	B-4	+	+	+
Hyphomicrobium sp.	854	–	+	+
Hyphomicrobium sp.	W54	–	–	–
Hyphomicrobium sp.	ZV-580	–	+	+
Hyphomicrobium sp.	B-522	–	+	+
Hyphomicrobium sp.	SW-809	–	+	+
Hyphomicrobium sp.	NQ-521gr	–	+	+
Tetramicrobium sp.	F-1	–	+	+

[a] Done in collaboration with P. Hirsch, Inst. Für Allgemeine Mikrobiologie, der Universität Kiel, 2300 Kiel, W. Germany.
[b] Fe- and Mn-containing media described in (69).
[c] EM observations of RR staining in thin sections (see 70).

extracellular polymers with metal deposition. They also emphasize that Mn-depositing ability is not simply associated with the presence of acidic polymers as Fe-depositing activity apparently is.

An interesting byproduct of this ultrastructural survey involved one strain (SW-809) that previously was found to avidly deposit Fe in a seawater medium (88). EM thin sections of this strain (Figure 6) revealed particles of the iron mineral lepidocrocite in close association with extracellular polymers. The lipidocrocite formed in the seawater medium and accumulated in large flocs containing very long bacterial hyphae. Apparently the mineral was bound nonspecifically by bacterial extracellular polymers in the flocs.

In other EM studies, MacRae and coworkers (114, 115) showed that washed cells of *Caulobacter, Micrococcus, Pseudomonas, Mycobacterium, Escherichia, Klebsiella, Corynebacterium,* and *Acinetobacter* nonspecifically precipitated positively charged ferric-iron sol. The coating of colloidal iron on cells was sufficiently dense to visualize Fe-encrusted whole cells in the EM without the usual negative-contrast reagents. The authors did not speculate on the mechanism of adsorption of colloidal iron; however, because positively charged colloidal iron preparations similar to theirs commonly are employed by histologists for specific staining of acidic extracellular polymers (86), it may be assumed that the adsorption of colloidal iron probably was associated with acidic polymers on the surfaces of the cells.

These ultrastructural findings, along with considerations of Fe and Mn chemistry in natural waters (126), suggest that, under neutral or slightly acidic conditions, any bacteria that produce acidic extracellular polymers will non-

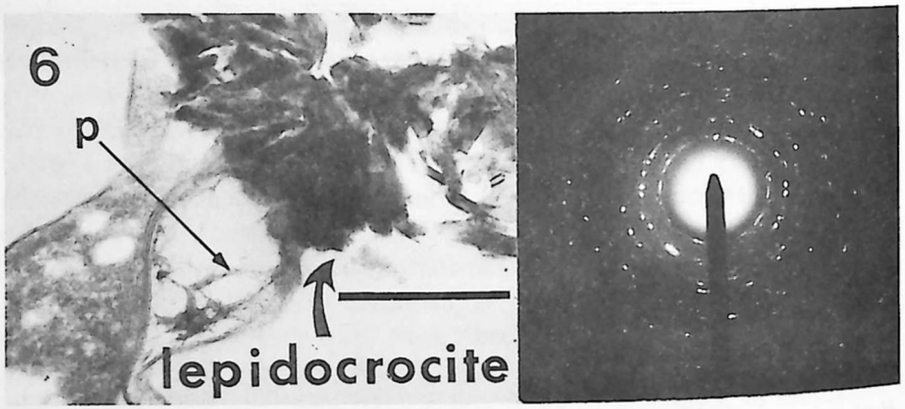


Figure 6 TEM thin section (*left*) showing extracellular polymers (*p*) of a hyphal budding bacterial cell (strain SW-809, see Table 2 and Ref. 88) associated with a large electron-dense particle identified as lepidocrocite by electron diffraction (*right*). Lepidocrocrite, an iron-hydroxide mineral, was formed in cultures growing in an Fe-containing seawater medium after two weeks of incubation. Particles of the mineral accumulated continuously thereafter in flocculent masses containing cells with very long hyphae. Bar = 0.5 μm. (Work done in collaboration with P. Hirsch).

specifically adsorb positively charged iron hydroxides that occur under these conditions. This proposed mechanism does not apply to Mn oxides, however, that are negatively charged above pH 6 (125, 132). Alternatively, I suggested (65) that Mn^{2+} may first bind to acidic polymers and then be oxidized biologically afterwards. Such a mechanism has been proposed to explain the formation of Mn oxide in association with extracellular polymers of *Pedomicrobium* spp. (70). Further considerations of ferromanganese-oxide surface chemistry suggest that once the oxide is formed, further binding and oxidation of the metals could occur autocatalytically (132).

Sheaths

SPHAEROTILUS NATANS Sheath structure in *Sphaerotilus natans* has been reviewed recently (40, 184). Ultrastructural, chemical, and fluorescent immunological studies have shown that the sheath consists of a polysaccharide-peptide-lipid complex, which is probably synthesized at the surface of cells. The sheath can vary in thickness depending on nutritional conditions. In addition to sheath polymers, *S. natans* also produces slimes of acidic polysaccharides that contain equimolar amounts of fucose, glucose, galactose, and galacturonic acid (62).

Whole sheaths of *S. natans* grown in the absence of excess Fe appear smooth in the EM (185). In thin section of these sheaths, fine fibrilar layers are seen (90, 142). The inner layers of the *S. natans* sheath appear to directly contact the outer membrane of the gram-negative cell envelope, suggesting that sheath material is synthesized and excreted at the surface of the cell envelope (40, 90). In apparent conflict with this idea is the finding that immunologically reactive new sheath material occurred only at the ends of growing *S. natans* filaments (151). This apparent discrepancy may be explained if it is assumed that sheath formation takes place during elongation of cells at the ends of filaments (40).

The sheath complex of *S. natans* can become encrusted with iron hydroxide when cells are cultivated in media containing excess chelated Fe (185). Iron hydroxide deposition is generally thought to be a passive process, probably similar to the nonspecific Fe deposition discussed above, but the possibility of active Fe oxidation has not been investigated. Manganese oxide is not deposited by *S. natans* under any conditions. In fact, any strain of *S. natans* that demonstrates Mn-depositing ability would be classified as *Leptothrix* spp. (25). No ultrastructural studies of Fe-encrusted *S. natans* sheaths in thin sections have been reported in the literature. This leaves an obvious void for those wishing to compare sheath structures in *S. natans* with those of *Leptothrix* spp., which have only been studied in metal-encrusted cells.

LEPTOTHRIX SPP. Little is known of the chemical composition or the underlying organic matrix structure of *Leptothrix* sheaths. The presence of acidic exopolysaccharides in addition to sheath polymers may be assumed from

numerous cultural observations (185), but the composition of the putative polysaccharides is unknown. A lack of information on *Leptothrix* sheath polymers was noted by Rouf & Stokes (155) when they first isolated their *Sphaerotilus discophorus* strains in 1964. [These strains are now assumed to be either *Leptothrix cholodnii* (185) or *L. discophora* (55)]. However, this situation has not been remedied in the 20 years since (130). Some EM observations of whole *Leptothrix* sheaths in cultures and natural samples have been published (14, 61, 185), but only limited information on sheath composition and ultrastructure has been available until now.

Preliminary study on the composition of the sheath of one of Stokes's strains of *Leptothrix* (*S. discophorus* J2R) was conducted by J. Makemson, then at American University of Beirut (personal communication). In these studies, sheaths of *Leptothrix* grown in media without added Fe or Mn contained 10–20% protein based on dry weight. The procedure used to purify sheaths was similar in principle to the lysis-washing procedure used to determine sheath composition of *S. natans* (152). No other chemical analyses of the organic composition of *Leptothrix* sheaths are found in the literature for comparison.

Recently, *Leptothrix* sheath ultrastructure has been investigated in natural samples from iron springs (27) and shallow ponds (67, 68, 73; Figures 1 and 2). TEM thin sections of conventionally fixed and embedded samples revealed that metal-encrusted sheaths and holdfasts of *Leptothrix* consist of fibrillar (Figure 1) or hexagonal matrices (27). Metals probably impregnate the organic matrix, rendering it electron-dense. In our investigations of *Leptothrix* (Figure 1), heavily encrusted sheaths rarely showed any evidence of the organic matrix, suggesting that metal oxides probably made up a large proportion of the material in the sheath. Thin sections of acidified leukoberbelin blue-extracted, RR-stained material (Figure 2) showed threads of RR-stained acidic polymers contacting protuberant areas of the cell envelope. These observations suggest that the sheath of *Leptothrix* spp. is composed of a metal-impregnated organic matrix that, like *S. natans* sheaths, may be synthesized and excreted at contact points along the surface of the cell.

Additional investigations of a newly isolated *Leptothrix discophora* strain (SS-1) in slide cultures showed that Mn oxide was deposited in sheath material at the growing tips of filaments that grew by elongation of individual cells (66). Thin sections of this strain grown without added Mn (Figure 3) revealed little of the sheath structure. Only fibrils of disorganized material (*P*, Figure 3), which could be unorganized sheath polymers, were seen. When Mn was added to the growth medium, attachment of Mn oxides to the outer membrane was detected (*arrows*, Figure 4). Further studies of sheath structure in strain SS-1 were impossible, because this strain ceased producing a structured sheath shortly after its isolation. However, it continued to produce unorganized extracellular polymers and maintained the ability to oxidize Mn^{2+}.

In contrast, *Leptothrix* cultures studied by Siebke (163) apparently maintained the ability to produce a structured sheath when growing in artificial media (131). Siebke's micrographs of strain 1120 show sheath matrix material in a layer inside a layer of Mn oxides. It is possible that in this strain the Mn oxides were deposited primarily in the outer part of the sheath in association with acidic exopolysaccharides, as described in *Pedomicrobium* spp. (70).

Based on the foregoing evidence, it is reasonable to conclude that sheaths of *Leptothrix* spp. may be similar in chemical composition and structure to those of *S. natans*. However, fundamental information on chemical composition and organization of the sheath polymers and on the relationship of the polymers to metal oxides in them is lacking.

Capsule of Siderocapsaceae

Encapsulated unicellular Fe- and Mn-depositing bacteria, the *Siderocapsaceae*, are encountered frequently in aquatic environments (46, 108), but few of them have been isolated in axenic culture (81). Of those that have been isolated, only members of the genus *Siderocapsa* have been studied by EM (46, 158). In none of these has the extracellular capsular material been analyzed chemically.

Dubinina & Zhdanov (46, 199) isolated two strains (A and G) of *Siderocapsa* spp. from meromictic lakes in Karelia in the Soviet Union. These strains deposited Fe and Mn in capsular material when grown in media containing these metals. The cells stained gram-negatively and were polarly flagellated. Both strains underwent considerable morphological variation in slide cultures, first forming rods and then cocci typical of *Arthrobacter* spp. Therefore, they were named *Arthrobacter siderocapsulatus*. Furthermore, based on these observations, Dubinina & Zhdanov (46) concluded that all *Siderocapsa* spp. probably belong to the genus *Arthrobacter*. Unfortunately, no thin sections were shown that would have revealed the wall structure and its association with the metal-encrusted polymers. Lacking evidence on wall structure, it may be concluded that the strains could be encapsulated *Arthrobacter* spp., but a negative gram reaction and polar flagella indicate other possibilities.

The ultrastructural studies of *Siderocapsa geminata* by Schmidt (158) clearly show that the polarly flagellated isolates possessed gram-negative envelopes. In many instances, the outer membranes of the cells extend to contact metal-impregnated capsular material in a manner similar to that observed in *Leptothrix* (Figures 1, 2, and 4). Furthermore, in thin sections of particulate material from the lake where Schmidt's isolates originated, an abundance of metal-encapsulated *Siderocapsa* cells with gram-negative cell envelopes was found. According to Schmidt's data the isolated *Siderocapsa* strains could be either *Vibrio* sp. or *Pseudomonas* sp. Additional work to distinguish between the two possibilities was inconclusive, suggesting that Schmidt may have been working with mixed cultures. Nonetheless, his EM work

clearly indicates, contrary to Dubinana & Zhdanova's conclusion (46), that his *Siderocapsa geminata* isolates were not *Arthrobacter* spp.

Stalk of Gallionella

Ehrenberg (47) first described the twisted, iron-encrusted stalk structures of *Gallionella* in ochre deposits and suggested they were fossil "infusoria." He named them after B. Gallion, an eighteenth-century French zoologist (198). The true nature of the structures called *Gallionella* was not discovered until Cholodny (32) showed that bean-shaped bacteria were delicately attached to the ends of the twisted stalks. Based on careful microscopic observations, Cholodny proposed that the bacteria secrete the twisted stalk as they grow. Since then the life cycle of *Gallionella* has been the object of some debate (82); however, in spite of recent claims that the stalk contains living mycoplasmoid organism (9, 10), Cholodny's concept of *Gallionella* has gained acceptance in most circles (however, see 87, 147).

Electron-microscopic investigations of *Gallionella* consist mainly of descriptions of whole-mounted stalks (9, 13, 80, 140) and attached apical cells (8, 87, 122, 127, 186). Studies of *Gallionella* in thin sections are rare, consisting of only two reports by Balashova (9, 11). Considerably more attention has been focused on the structure of stalks than on apical cells. Stalks consist of ribbon-like bands of iron-impregnated electron-dense fibrils eminating from one side of oval or bean-shaped apical cells (82, 128, 186, 191, 194). Actively growing *Gallionella* colonies contain numerous branched stalks with apical cells at the ends of each branch (32, 82). Binary fission of the apical cells gives rise to the branched filaments (81). These results allow for the generally accepted conclusion that *Gallionella* apical cells excrete stalk material, which becomes encrusted with iron hydroxide as they grow.

Recently, *Gallionella* in samples from the metalimnion of a lake in Norway were analyzed in a microprobe (87). Apical cells contained the usual elements expected in living bacteria, whereas stalk material and electron-dense particles associated with stalks contained large amounts of Fe and traces of Si, S, Cl, K, Ca, and Cu, but no P. The lack of P indicated that these structures consisted of nonliving material. This evidence was given in direct rebuttal to Balashova's proposal (9, 10) that a *Gallionella* stalk consists of a living mycoplasmoidal organism that reproduces by means of electron-dense "elementary bodies."

It should be noted that in presenting her evidence for mycoplasmas in *Gallionella* stalks, Balashova (9) offered some excellent electron micrographs of the *Gallionella* stalk fibrils. Her micrographs show broken, Fe-encrusted filaments containing very fine (10–20 nm) fibrils in their core. The tenuous mycoplasma theory aside, Balashova's work supports the notion first derived from micrographs of Vatter & Wolfe (186) that the stalk of *Gallionella* consists of Fe-encrusted fibrils excreted by the apical cells.

The organic composition of *Gallionella* stalk fibrils is uncertain. Both Hanert (80) and Balashova (9) gave evidence (UV absorption and protease digestion, respectively) that the fibrils may contain protein, but their experiments are not convincing because results with control specimens were not given. Diffraction studies and NMR spectroscopy demonstrated that amorphous ferric hydroxide in the stalk probably is covalently bound to nonpolar groups in the filaments (118). Thus, it appears that stalk fibrils may contain protein, but given the appearance of the zone of stalk attachment to apical cells (Figure 7; see also Figure 5 in reference 128), the involvement of other polymers cannot be ruled out.

Figure 7 shows that the apical *Gallionella* cell possesses a gram-negative cell envelope. A layer of fine fibrilar material is present in the zone of stalk attachment. Because this thin section was not poststained, structures inside the cell are not well defined; however, the stalk fibrils are very electron-dense and well defined, presumably because they were impregnated with iron hydroxide.

Before now the only published thin sections of *Gallionella* cells were those of Balashova & Cherni (11). In their study, one tangential section showed electron-dense, ring-like structures within the cytoplasm that were identified as spherical bodies comparable to forms seen in cross sections of stalk filaments. It was concluded that both forms represented the mycoplasmoid organism of *Gallionella*. In my opinion, these speculative interpretations have created some

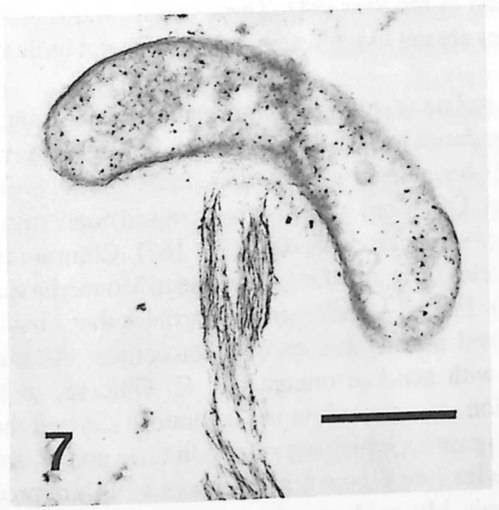


Figure 7 TEM thin section showing the zone of stalk attachment on a *Gallionella* apical cell. Note the area of fine fibrillar material near the surface of the cell and electron-dense stalk fibrils originating from it. Structures are not well defined inside the cell because no poststaining was used. However, a gram-negative envelope and some internal structures are evident. Bar = 0.5 μm.

unnecessary confusion concerning the biology of this fascinating organism (for example, see reference 147).

Metallogenium

Metallogenium particles are frequently observed in natural environments at aerobic-anaerobic interfaces where Fe and Mn are deposited (108, 109, 140, 197). Microfossils resembling *Metallogenium* also have been described in precambrian rocks (32a; see additional references in 55, 106, 119, 135a, 135b, 197); however, the origins of the *Metallogenium* forms are not clear (75, 135, 135a, 135b, 162).

Recent EM work has helped to elucidate the *Metallogenium* story, at least partially. Examination of thin sections of *Metallogenium* particles in samples from several different lakes have failed to reveal biological membranes or other structures normally associated with living cells (75, 94, 102, 158). Furthermore, acridine orange–stained particles from Lake Washington did not fluoresce, indicating a lack of nucleic acids (75). Electron-microprobe analyses of these particles indicated that they contained nearly 70% manganese oxide, 11% water, and lesser amounts of other elements, including Fe, P, and S. Because the electron microprobe could not detect elements lighter than Na, C and N could not have been detected in these analyses. Similar studies of *Metallogenium* particles in Lake Léman, Switzerland revealed that bacterial cells adhered to the particles and that their mineral composition varied with depth and season of the year (94). These studies indicate that *Metallogenium* particles in lakes are not living forms, but they do not indicate what their origin is.

Attempts to isolate *Metallogenium* organisms from samples containing the particles in abundance have failed (75, 158). However, several workers (106, 128, 158, 162) have reported *Metallogenium* particles in cultures of Mn-depositing fungi. Questions have also been raised concerning the origins of the fungal *Metallogenium* particles (106, 128, 162). Commonly these Mn-oxide–containing particles form near fungal hyphae in Mn media that contain starch or agar (126, 162). Fungal *Metallogenium* particles that I have examined in thin sections contained no membranes or other cellular structures; nor did such particles stain with acridine orange (W. C. Ghiorse, unpublished observations). In addition, recent work in my laboratory showed that *Metallogenium*-like particles (Figure 8), which are similar in form and in staining properties to the fungal particles (see Figure 8 in reference 128), are produced in agar gels when a diffusable Mn-oxidizing factor excreted by *Leptothrix* sp. oxidizes Mn^{2+} in the gel.

These observations suggest that the fungal *Metallogenium* particles may also result from oxidation of Mn by diffusible Mn-oxidizing factors in agar or in starch polymers in media where the fungi grow. If true, this explanation for the fungal *Metallogenium* particles could create problems concerning the identity of the organisms that constitute the genus *Metallogenium* (198), especially *M. symbioticum* that has only been obtained in cocultures with fungi. Most questions concerning the identity of *Metallogenium* particles in fungal cultures can be answered by careful microscopic work and chemical analysis. However, questions concerning the identity of *Metallogenium* organisms originally described by Perfil'ev & Gabe in capillaries (140; see plate 4.4 and Figures 3–9 in reference 25) cannot be answered without repeating the original work. Ideally, such a reinvestigation would include fluorescence microscopy and TEM of thin sections.

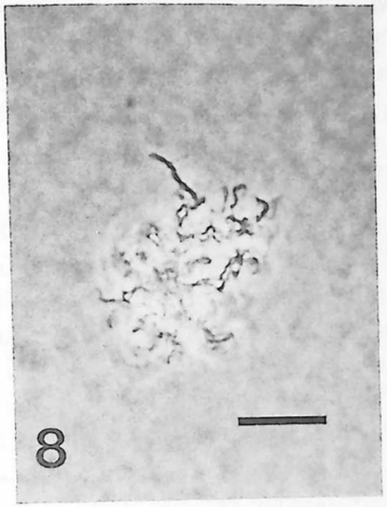


Figure 8 Phase contrast image of a *Metallogenium*-like particle that formed in an agar gel as a result of Mn^{2+} oxidation by a diffusible Mn-oxidizing factor produced by *Leptothrix* sp. Numerous particles such as this were found in a band of Mn oxide that formed several millimeters away from a well containing the *Leptothrix* cells. Bar = 10 μm. (Work done by A. M. Ress).

PHYSIOLOGICAL ASPECTS OF FE AND MN DEPOSITION

Since the time of Winogradsky (190) and Beijerinck (15), speculation on the physiological role of Fe and Mn deposition in bacteria has generated considerable interest and caused some controversy. The speculation and controversy have been stimulated by indications that Fe^{2+} and Mn^{2+} may be used as energy sources by bacteria and evidence that a variety of enzymatic and nonenzymatic mechanisms may exist, especially for Mn oxidation. Much of the evidence has

been discussed previously—in some cases in great detail—in monographs, review articles, and book chapters (40, 55, 113, 119, 128, 135, 185, 191, 194). In the following discussion, therefore, previously reviewed information will be summarized briefly, and new information will be discussed in greater detail.

Chemoautotrophy in Gallionella

Gallionella should probably be considered a chemoautotroph, or at least a chemolithotroph (96a), based on its ability to grow in inorganic media, its uptake of $^{14}CO_2$ in cultures, its requirements for O_2 and Fe^{2+} for growth, and its deposition of substantial amounts of $Fe(OH)_3$ during growth (55, 82). In addition, Hanert and coworkers (personal communication) recently found evidence that their cultures of *Gallionella* fix CO_2 via ribulose-bisphosphate carboxylase. This evidence notwithstanding, rigorous proof of chemoautotrophy in *Gallionella* is still lacking. In fact, very little is known of *Gallionella* nutrition and physiology, possibly because axenic cultures have not been easily obtained.

Fe and Mn Oxidation by Heterotrophic Bacteria

It is possible that some heterotrophic Fe- and Mn-depositing bacteria that can grow under microaerophilic conditions similar to those that encourage the growth of *Gallionella* may oxidize Fe enzymatically. It is also possible that such bacteria may derive some useful energy from Fe oxidation. However, evidence supporting these possibilities is lacking. In fact, with a few exceptions (149, 150), possible Fe oxidation by heterotrophic bacteria has generally not been investigated because Fe autoxidizes rapidly under aerobic conditions at neutral pH. Under microaerophilic conditions, however, autoxidation of Fe^{2+} would be expected to proceed at slower rates, especially if slightly acidic conditions (pH 5.0–6.0) prevailed. Under such conditions, biological Fe oxidation mechanisms may become more significant. The latter possibility has been recognized by most researchers, but few, if any, investigations have been carried out to demonstrate biological mechanisms of Fe oxidation under these conditions.

On the other hand, investigations of bacterial Mn oxidation are numerous. In this case, biological catalysis is more likely to be important in natural environments because Mn^{2+} does not autooxidize rapidly below pH 9 (170). Ehrlich (57) reviewed and summarized the different forms of bacterial Mn oxidation that have been reported. He concluded that many unrelated bacteria can mediate Mn oxidation via either enzymatic or nonenzymatic oxidation mechanisms. Further, he concluded that the chemical mechanisms are not the same in all cases. It should be pointed out, however, that in many of the cases cited by Ehrlich (57), the Mn^{2+} oxidation mechanisms were directly related to defenses against O_2 toxicity or respiratory activity.

DEFENSE AGAINST O_2 TOXICITY Not all enzymatic Mn^{2+} oxidations should be expected to yield useful energy to the organisms that catalyze them. For example, based on studies of *Leptothrix pseudoochraceae, Arthrobacter siderocapsulatus,* and *Metallogenium personatum,* Dubinina (44, 45) proposed that Mn^{2+} and Fe^{2+} may be oxidized enzymatically by reaction with small amounts of H_2O_2 produced during aerobic growth of the bacteria on glucose or other organic substrates. In her proposed mechanism, catalase mediates the peroxidatic reaction: $Mn^{2+} + H_2O_2 \rightarrow MnO_2 + 2H^+$. This reaction would not be expected to yield useful energy to the cells, but would rid them of toxic H_2O_2. In addition, Dubinina (44, 45) proposed that once MnO_2 was formed, it could be reduced by excess H_2O_2, destroying H_2O_2 chemically.

A similar role for Mn in cellular defenses against O_2 toxicity has been found recently in *Lactobacillus plantarum*. In these bacteria, mM concentrations of intracellular Mn^{2+} appear to replace μM concentrations of superoxide dismutase (3). An O_2^--destroying mechanism was proposed in which Mn^{2+} is oxidized to Mn(III) by O_2^-. The Mn(III) formed could then be reduced by NADPH, reforming Mn^{2+} for further reaction with O_2^-. In addition to elevated intracellular Mn concentrations, *L. plantarum* also has been found to contain a Mn(III)-containing pseudocatalase (104).

The significance of these findings is emphasized by the fact that Mn- and Fe-containing enzymes are frequently involved in biological reactions with O_2 and the toxic products of its metabolism. This probably reflects a close linkage in the chemistry of these three elements. With this in mind, it should not be surprising to find that biological transformations of Fe and Mn occur in association with bacterial respiratory activity.

These considerations of O_2 toxicity invite further speculation on environmental aspects of Fe- and Mn-deposition by bacteria, such as *Leptothrix* spp., that deposit ferromanganese oxide in extracellular polymer complexes. If H_2O_2 were produced in the periplasmic space of these bacteria during oxidative metabolism, it might diffuse outward and be eliminated extracellularly by either enzymatic oxidation or nonenzymatic reduction. If such a mechanism were operating under oligotrophic and microaerophilic conditions, low levels of H_2O_2 would be produced under these conditions and could participate in peroxidase-oxidation and subsequent deposition of metal oxides. On the other hand, under fully aerobic conditions with excess organic nutrients, excess H_2O_2 would be produced, and reduction of metal oxides would be expected.

While substantial evidence supporting a role for Mn in defending Fe- and Mn-depositing bacteria against H_2O_2 toxicity is lacking, it has been shown that growth-limiting amounts of H_2O_2 are produced by *Leptothrix pseudochracea* (45) and *L. discophora* SS-1 (W. C. Ghiorse, unpublished observations) during growth at elevated glucose concentrations. Furthermore, reports of Mn-oxide reduction during aerobic growth of *Leptothrix* spp. (45, 185) and *Arthrobacter* sp. (23) in heterotrophic media lend strong support to the idea.

INVOLVEMENT OF RESPIRATORY SYSTEMS Enzymatic Mn oxidation related to respiratory activity of bacterial cells has been observed by several workers, but few attempts have been made to investigate details of the enzyme systems involved. In one such investigation, Hogan (91) studied enzymatic oxidation of Mn^{2+} in whole cells and cell-free extracts of *Sphaerotilus discophorus* (*Leptothrix* spp.). She noted that most of the activity in extracts was associated with 48,000-xg and 100,000-xg particulate fractions (91, 123). In these experiments, Mn-oxidizing activity was estimated by measuring O_2 uptake in the presence and absence of Mn^{2+}. The membrane-associated Mn-oxidizing activity as well as endogenous O_2 uptake were inhibited by cyanide, azide, and o-phenathroline, suggesting that cytochromes or other metalloenzymes were involved. Unfortunately, the Mn-oxidizing activity in fractionated extracts was not further characterized nor was it related to Mn oxidation by whole cells. Therefore, definite conclusions concerning the biological significance of these experiments cannot be reached.

Enzymatic Mn oxidation in bacterial cell extracts was also studied by Douka (42, 43). The activity was sensitive to $HgCl_2$ and heat. Enzyme kinetics were reported, but components of the system were not studied.

Ehrlich (56) also investigated components of an enzymatic Mn-oxidizing system in cell extracts of several bacteria from the Galapagos vent samples. In one instance Mn-oxidizing activity was inhibited by several different cytochrome inhibitors. Cytochrome c was reduced by Mn^{2+} in particulate fractions only when a soluble periplasmic factor was added back. Significantly, ATP was synthesized from ADP and Pi during Mn^{2+} oxidation by membrane vesicles, suggesting that the bacteria could couple Mn oxidation to ATP synthesis.

By far the most detailed studies of bacterial enzymatic Mn oxidation are those of Ehrlich and coworkers on isolates from deep-sea Mn nodules and sediments (for summary of this work see 53, 55, 57). The bacteria include *Arthrobacter* sp., *Oceanospirillum* sp., and *Vibrio* sp., which oxidize Mn^{2+} only when ferromanganese oxide or MnO_2 are present initially. Because the Mn oxides are very insoluble, the experimental systems are complicated by the constant presence of MnO_2 particles. Experiments with *Arthrobacter* sp. showed that maximum oxidation rates occurred at an optimal ratio of cells or extract to MnO_2 (49), suggesting that the binding of both cells and Mn^{2+} to the insoluble MnO_2 was important in the oxidation mechanism. In all of these bacteria the Mn-oxidizing activity was O_2-dependent. Also, in general, the activity was sensitive to inhibitors of respiration, including rotenone, dicumarol, antimycin A, NOQNO, azide, and cyanide (57). More detailed experiments with fractionated cell extracts of *Oceanospirillum* sp. and *Vibrio* sp. showed that Mn-oxidizing activity required both the particulate fraction that contained cell membranes, a heat-stable soluble periplasmic factor, and MnO_2.

Mn^{2+} was shown to reduce cytochrome c in the membrane fraction only when the heat-stable factor and MnO_2 were present, and ATP synthesis occurred during Mn oxidation only when membrane vesicles and MnO_2 were present.

The presence of MnO_2, which may add reactive surfaces to the experimental systems described above, introduces difficulties in the interpretation of some of the results obtained from them. However, despite these difficulties, the work of Ehrlich and coworkers allows for the following conclusions: (*a*) A variety of heterotrophic bacteria possess enzymes capable of mediating Mn oxidation on the surface of the MnO_2 particles. (*b*) Many of the Mn-oxidizing enzyme systems involve respiratory enzymes. (*c*) ATP synthesis can be coupled to Mn oxidation by membrane vesicles in these systems, suggesting that some of the bacteria may be able to derive useful energy from the Mn oxidation.

OTHER MN-OXIDIZING SYSTEMS Most of the enzymatic Mn^{2+} oxidizing systems described above appear to involve direct interaction of Mn^{2+} with enzymes that defend against O_2 toxicity or with enzymes in energy-generating, respiratory systems. However, Ehrlich and coworkers (195, 200) recently described a different type of bacterial Mn-oxidizing system that did not directly involve either of these types of enzymes. In this system, Mn-oxidizing activity was inhibited by heat, mercury compounds, and protease treatments. The activity in cell extracts displayed a temperature optimum at 30°C and a pH optimum at 7.0; however, it was not affected by rotenone, antimycin A, NOQNO, or sodium azide. Difference spectra indicated that the usual complement of cytochromes and possibly a flavoprotein were present in the extracts. Fractionation studies and partial purification by gel chromatography suggested that a low-molecular-weight, periplasmic factor, which was heat-stable and nondialysable, and an intracellular enzyme were involved in Mn oxidation. The activity in periplasmic fractions was not inhibited under N_2, whereas the activity in sonicates was reduced by 50%. Reconstitution of intracellular and periplasmic fractions of spheroplasts resulted in greatly increased activity, suggesting that the enzyme and periplasmic factor operated together to oxidize Mn^{2+}.

To explain the foregoing findings, Zindulis & Ehrlich (200) proposed that Mn^{2+} was oxidized by the low-molecular-weight factor in the periplasm of the cells. The reduced form of the periplasmic factor was not directly oxidized by O_2; rather, it was assumed to be reoxidized by the intracellular enzyme associated with the cell membrane. Finally, the intracellular enzyme was reoxidized by O_2. It should be noted that the periplasmic factor in this system behaved in many respects like an intracellular Mn-oxidizing protein found in *Pseudomonas manganoxidans* (96). However, no enzymatic Mn-oxidizing activity was found in the *P. manganoxidans*. Thus, the two systems appear to be different.

Mn-oxidizing factors that behave like proteins have also been reported in *Leptothrix* spp. (185). Recent studies in my laboratory have shown that the factor(s) of *L. discophora* SS-1 are sensitive to heat, azide, $HgCl_2$, and protease treatment (L. F. Adams, unpublished observations). Apparently the factors are excreted into the medium by *L. discophora* SS-1 and probably by other *Leptothrix* spp. as well.

Nonenzymatic Mn binding and oxidation also has been reported recently in mature spore suspensions of a marine *Bacillus* sp. (100, 153). The activity was inhibited by elevated temperature, low pH, EDTA, azide, cyanide, and $HgCl_2$, but not by aldehydes, ethylene oxide, or UV irradiation. The data indicated that Mn^{2+} was complexed by a spore-coat protein and then rapidly oxidized (153).

Possible Mn Mixotrophy and Other Effects of Mn

Based on his demonstration of ATP synthesis coupled to Mn^{2+} oxidation by bacterial membrane vesicles, Ehrlich (52, 53, 57) assumed that his Mn-oxidizing bacteria can use energy liberated during Mn^{2+} oxidation to support mixotrophic growth. While Ehrlich's evidence is compelling at the biochemical level, as yet, no evidence for Mn-stimulated growth of whole bacteria has been demonstrated.

Previous claims for Mn mixotrophy and chemoautotrophy in *Sphaerotilus discophorus* (*Leptothrix* sp.) by Ali & Stokes (2) were based on Mn stimulation of growth in media containing little or no added organic nutrients. These results have been disputed (78) on the basis of probable Mn^{2+} interference with the protein and DNA determinations used. However, Ehrlich (55) pointed out that, even when corrections were made for Mn^{2+} interference, the original data show Mn-stimulated growth.

More recently, claims were made for Mn mixotrophy and chemoautotrophy in a marine pseudomonad (99). These were based on preliminary observations of Mn-stimulated growth in chemostat culture. These findings have not been substantiated, but they are further indications that Mn mixotrophy may exist in bacteria. On the other hand, there are few indications that Mn chemoautotrophy exists, because CO_2 fixation coupled to Mn stimulation of growth has not been demonstrated (135b).

It is important to note in discussing Mn stimulation of growth that Mn^{2+} and Fe^{3+} may also inhibit growth of *Sphaerotilus* and *Leptothrix* spp. (1, 30, 78, 95, 127, 144, 185). The common observation of Mn inhibition of growth raises questions concerning possible toxicity of Mn^{2+} and other physiological roles of this cation that may affect intracellular enzymes and bacterial transport systems in unknown ways. Except for one report indicating that Mn^{2+} and Mg^{2+} stimulate PHB-utilizing enzymes in *S. discophorus* (*Leptothrix* sp.) (169), effects of Mn^{2+} on intracellular enzymes of Mn-depositing bacteria have not been investigated. Because it appears that Mn active transport systems probably are found in all bacteria (164), it is reasonable to assume that Mn can be transported specifically by Mn-depositing bacteria. Although excessive Mn^{2+} may inhibit bacterial growth, it is possible that certain concentrations of Mn^{2+} may stimulate growth by unknown physiological interactions with specific intracellular enzymes. Such considerations should be taken into account when results of experiments showing Mn-affected growth are interpreted.

GENETICS OF FE AND MN DEPOSITION

Comparatively little information is available on genetic aspects of Fe- and Mn-depositing bacteria. Even *Thiobacillus ferrooxidans*, which is relatively well characterized biochemically, is poorly understood genetically (171). Except for recent work demonstrating the existence of cryptic plasmids in *T. ferrooxidans* and indications of phenotypic variations in their ability to use inorganic and organic energy sources (117, 121), virtually nothing is known of the genetics of these or other acidophilic, Fe-depositing chemoautotrophs.

The genetic aspects of metal deposition by other Fe- and Mn-depositing bacteria is also poorly understood. Most of the available information is limited to reports of spontaneous and irreversible loss of Mn-depositing activity, which frequently is observed after prolonged storage of cultures (69, 76, 112, 185). In some cultures of *Leptothrix* spp., loss of Mn-depositing ability is accompanied by loss of sheath-forming ability or change from rough to smooth colony appearance (185), but such variations do not occur in all *Leptothrix* spp. For example, *L. discophora* apparently is more stable with respect to Mn-depositing ability than other *Leptothrix* species. Some strains of *L. discophora* have been reported to maintain this trait after ten years in laboratory culture (185). In my laboratory, *L. discophora* strain SS-1 (Figures 3 and 4) still deposits Mn after five years in laboratory cultures, but it has lost the ability to form morphologically distinct sheaths (W. C. Ghiorse, unpublished observations).

Very recently, plasmid-associated Mn-depositing activity was reported in studies of heterotrophic Mn-depositing freshwater (76) and marine bacteria (112). In both instances, spontaneous loss of plasmids was correlated with loss of Mn-depositing activity after long-term storage of cultures. In one of the marine bacteria (112), the presence of a plasmid ($M_r = 24 \times 10^6$) was correlated with increased levels of $^{54}Mn^{2+}$ binding and Mn-oxide deposition, indicating that some functions related to Mn-binding and oxidation were plasmid-encoded. On the other hand, in the same study plasmid screening of several other cultures containing Mn-depositing and non–Mn-depositing pairs showed no uniform positive correlation between the presence of a plasmid and the ability to deposit Mn. In fact, one half of the Mn-depositing and non–Mn-depositing pairs examined contained no detectable plasmids. Like Fe

oxidation in *T. ferrooxidans* (121), Mn-depositing activity probably is encoded on chromosomal genes in these bacteria.

Recent work in my laboratory (L. F. Adams & R. Lynfield, unpublished observations) showed that *Leptothrix discophora* SS-1 also contained no plasmids detectable by three different lysis and plasmid isolation procedures. These procedures were able to detect large and small plasmids in *Escherichia coli* V517 (116), *Bacillus* SG-1 (137; provided by S. D. Chapnick), *Streptococcus faecalis* DS5 (provided by G. Dunny), and in several pseudomonads, including one containing the TOL plasmid ($M_r = 77 \times 10^6$) of *Pseudomonas putida* (60). Because *L. discophora* SS-1 invariably deposits Mn oxide on Mn-containing media, it can be concluded that its Mn-depositing ability also probably is encoded on chromosomal genes.

Although Mn-depositing ability is a stable property of strain SS-1, it may be significant that its ability to form a morphologically distinct sheath was lost after a few months of cultivation. One possible explanation for such a loss is that some genes for sheath formation may have been encoded on plasmids that were selected against by the artificial-culture conditions employed. As discussed above, isolated strains of *Leptothrix discophora,* like other *Leptothrix* spp. (185), appear to excrete protein-like Mn-oxidizing factor(s) into the growth medium. If the oxidizing factors, in fact, are proteins, then Mn-oxidizing activity of this strain may be controlled by one or a few chromosomal genes. In that case, studies on the genetic control of Mn oxidation by this strain and other *Leptothrix* spp. would be greatly simplified.

SUMMARY, CONCLUSIONS, AND FUTURE PROSPECTS

An attempt has been made to illuminate past and present problems of concern to those interested in the biology of Fe- and Mn-depositing bacteria. Past confusions concerning identity and nomenclature of some of these bacteria still persist (e.g. the identity of *Sphaerotilus discophorus* vs *Leptothrix* spp. and of *Metallogenium*), but these should not be problems for future workers who carefully consider information presently available in the literature.

Important questions concerning the activities of Fe- and Mn-depositing bacteria in natural environments and in laboratory cultures remain to be resolved, notably the importance relative to geochemical factors of Fe- and Mn-depositing bacteria, including *Leptothrix ochracea, Crenothrix polyspora, Clonothrix* sp., *Gallionella,* and members of the *Siderocapsaceae* as well as Fe- and Mn-depositing heterotrophs. Work on this problem will be aided by combined microbiological and geochemical studies, as well as studies of the bacteria in axenic cultures; however, so far, axenic cultures of many of the classical Fe- and Mn-depositing bacteria have not been available. This deficiency may be overcome by intelligent application of microaerophilic and gradient cultural methods that have been successful for enrichments of *Gallionella.*

On the other hand, many different heterotrophic Fe- and Mn-depositing bacteria have been isolated from environments containing ferromanganese deposits; but their importance in natural Fe and Mn deposition is also uncertain, and their metal-depositing mechanisms are not well understood. Some generalizations can be made, however, because many of them appear to grow under low-nutrient conditions and the majority are aerobes.

In a few cases, structures associated with Fe and Mn deposition have been studied by EM. Usually deposition of metal oxides occurs in association with extracellular polymers. Because bacterial polymers may be very common in natural environments, it follows that ferromanganese-oxide deposition in nature may occur in association with bacterial extracellular polymers. The polymers may consist of acidic polysaccharides, proteins, or polysaccharide-protein-lipid complexes that have been found in metal-encrusted bacterial structures. More chemical analyses of metal-depositing bacterial polymers are needed to understand the metal-polymer interactions.

Based on ultrastructural evidence and considerations of Fe and Mn chemistry under natural conditions (aerobic, neutral pH), a hypothetical mechanism for polymer-associated Fe and Mn deposition can be proposed. In this mechanism, free Mn^{2+} would bind to negatively charged polymers and then be oxidized in the polymer matrix, possibly by Mn-oxidizing enzymes or Mn-oxidizing factors excreted by the bacteria. Fe^{2+}, on the other hand, would autoxidize rapidly under the aerobic and neutral-pH conditions, requiring no biological intervention. Therefore, it is proposed that positively charged iron-hydroxide species [e.g. $Fe(OH)^{2+}$, $Fe(OH)_2^+$] formed during autoxidation would bind to the negatively charged polymers. In this hypothesis, Fe^{2+} could also be oxidized biologically by the same mechanism proposed for Mn^{2+} oxidation, but biological Fe^{2+} oxidation would not be important unless more acidic (pH 5–6) and microaerophilic conditions prevailed. Once ferromanganese-oxide deposition was initiated in the polymer matrix, abiological surface catalysis could accelerate the rate of deposition. Thus, biological and abiological catalyses both may be involved in the proposed deposition mechanism.

Studies with concentrated cell suspensions and cell extracts have demonstrated that many bacteria possess enzyme systems that catalyze Mn oxidation. Respiratory enzyme systems and enzymes involved in defense against O_2 toxicity are involved in some cases, but nonenzymatic Mn-oxidizing factors are also known. Some of the nonenzymatic factors appear to be Mn-oxidizing proteins, which may be located in the cell or spore envelope. In *Leptothrix* spp. a Mn-oxidizing protein appears to be excreted by the cells.

Evidence showing that ATP synthesis can be coupled to Mn oxidation indicates that bacteria may be able to derive useful energy from Mn oxidation. Thus, Mn mixotrophy is a distinct possibility. However, as yet, no unequivocal

evidence showing mixotrophic growth of Mn-depositing bacteria has been published. Even less evidence exists for Mn chemoautotrophy. Because Mn^{2+} is known to variously inhibit and stimulate growth of Mn-depositing bacteria, it may be concluded that the physiological effects of Mn^{2+} are quite variable. Therefore, studies showing Mn stimulation of growth should be interpreted with caution.

Finally, information on the genetics of Fe and Mn depositing bacteria is scanty. Spontaneous loss of Mn-depositing activity has been reported frequently in stored cultures of heterotrophic Mn depositors. In some *Leptothrix* spp. this loss may be accompanied by a loss of sheath-forming ability. Plasmids have been detected in some Mn-depositing bacteria, but usually Mn-oxidizing ability has not been associated with them.

The recent work indicating that Mn-depositing *Leptothrix* spp. probably produce Mn-oxidizing proteins suggests that Mn-oxidizing ability could be encoded on one or a few genes in these bacteria. If this is the case, then studies of the genetics of their Mn-oxidizing ability may be greatly simplified. In the future, genetic studies of such Fe- and Mn-depositing bacteria could form the basis for biotechnological applications of these bacteria in the areas of metal pollution control or metal recovery in the mining industry.

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Literature Cited

1. Adams, L. F., Ghiorse, W. C. 1982. *Abstr. Ann. Meet. Am. Soc. Microbiol.* N24:182
2. Ali, S. H., Stokes, J. L. 1971. *Antonie van Leeuwenhoek* 37:519–28
3. Archibald, F. S., Fridovich, I. 1981. *J. Bacteriol.* 145:442–51
4. Arcuri, E. J., Ehrlich, H. L. 1980. See Ref. 178, pp. 339–44
5. Arcuri, E. J., Ehrlich, H. L. 1983. *Z. Allg. Mikrobiol.* 20:583–86
6. Aristovskaya, T. V. 1961. *Dokl. Akad. Nauk SSSR* 136:111–14 (Engl. transl.)
7. Aristovskaya, T. V., Hirsch, P. 1974. See Ref. 25, pp. 151–53
8. Balashova, V. V. 1967. *Microbiology USSR* 36:541–44
9. Balashova, V. V. 1967. *Microbiology USSR* 36:879–81
10. Balashova, V. V. 1968. *Microbiology USSR* 37:590–98
11. Balashova, V. V., Cherni, N. E. 1970. *Microbiology USSR* 39:298–302
12. Balkwill, D. L., Maratea, D., Blakemore, R. P. 1980. *J. Bacteriol.* 141: 1399–408
13. Beger, H., Bringmann, G. 1953. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. II Orig.* 107:305–17
14. Beger, H., Bringmann, G. 1953. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. II Orig.* 107:318–34
15. Beijerinck, M. W. 1913. *Folia Microbiol. Delft* 2:123–34
16. Beveridge, T. J., Meloche, J. D., Fyfe, W. S., Murray, R. G. E. 1983. *Appl. Environ. Microbiol.* 45:1094–108
17. Billy, C., Blanc, P. 1982. *C. R. Acad. Sci. Paris* 295:303–5
18. Blakemore, R. P. 1982. *Ann. Rev. Microbiol.* 36:217–38
19. Brierley, C. L. 1982. *Sci. Am.* 247:44–53
20. Bromfield, S. M. 1956. *Austr. J. Biol. Sci.* 2:238–52
21. Bromfield, S. M. 1974. *Soil Biol. Biochem.* 6:383–92
22. Bromfield, S. M. 1978. *Austr. J. Soil Res.* 16:91–100
23. Bromfield, S. M., David, D. J. 1976. *Soil Biol. Biochem.* 8:37–43
24. Bromfield, S. M., Skerman, V. B. D. 1950. *Soil Sci.* 69:337–48
25. Buchanan, R. E., Gibbons, N. E., eds. 1974. *Bergey's Manual of Determinative Bacteriology,* Baltimore: Williams & Wilkins. 1268 pp. 8th ed.
26. Burnett, B. R., Nealson, K. H. 1981. *Deep-Sea Res.* 28A:637–45
27. Caldwell, D. E., Caldwell, S. J. 1980. *Geomicrobiol. J.* 2:39–53
28. Callender, E., Bowser, C. J. 1976. In *Handbook of Strata-Bound and Stratiform Ore Deposits,* ed. K. H. Wolf, pp. 341–94. Amsterdam: Elsevier
29. Calvert, S. E., Price, N. B. 1977. In *Marine Manganese Deposits,* ed. G. P. Glasby, pp. 45–86. Amsterdam: Elsevier. 523 pp.
30. Chang, Y., Pfeffer, J. T., Chain, S. K. 1980. *Appl. Environ. Microbiol.* 40: 1049–52
31. Chapnick, S. D., Moore, W. S., Nealson, K. H. 1982. *Limnol. Oceanogr.* 27:1004–14
32. Cholodny, N. 1926. *Die Eisenbakterien: Beitrage zu einer Monographie. Pflanzenforschung.* Jena: Fischer. 162 pp.

32a. Cloud, P. E. 1965. *Science* 148:27–35

33. Colmer, A. R., Temple, K. L., Hinkel, M. E. 1950. *J. Bacteriol.* 59:317–28
34. Costerton, J. W., Geesey, G. G., Cheng, K. J. 1978. *Sci. Am.* 238:86–95
35. Crearar, D. A., Knox, G. W., Means, J. L. 1979. *Chem. Geol.* 24:111–35
36. Cullimore, D. R., McCann, A. E. 1977. In *Aquatic Microbiology,* ed. F. A. Skinner, J. M. Shewan, pp. 219–61. New York: Academic. 369 pp.
37. Dean, W. E., Ghosh, S. K. 1981. In *Geology and Geochemistry of Manganese,* ed. I. M. Varentsov, G. Y. Grasselly, 3:256–77. Budapest: Akademiai Kiado
38. Degens, E. T., Ittekkot, V. 1982. *Nature* 298:262–64
39. Degens, E. T., Watson, S. W., Remsen, C. C. 1970. *Science* 168:1207–8
40. Dondero, N. C. 1975. *Ann. Rev. Microbiol.* 29:407–28
41. Dorn, R. I., Oberlander, T. M. 1981. *Science* 213:1245–47
42. Douka, C. E. 1977. *Soil Biol. Biochem.* 9:89–97
43. Douka, C. E. 1980. *Appl. Environ. Microbiol.* 39:74–80
44. Dubinina, G. A. 1979. *Microbiology USSR* 47:471–78
45. Dubinina, G. A. 1979. *Microbiology USSR* 47:631–36
46. Dubinina, G. A., Zhdanov, A. V. 1975. *Int. J. Syst. Bacteriol.* 25:340–50
47. Ehrenberg, C. G. 1836. *Poggendorfs Ann. Phys. Chem.* 38:213–27
48. Ehrlich, H. L. 1963. *Appl. Microbiol.* 11:15–19
49. Ehrlich, H. L. 1966. *Dev. Ind. Microbiol.* 7:279–86
50. Ehrlich, H. L. 1968. *Appl. Microbiol.* 16:197–202
51. Ehrlich, H. L. 1975. *Soil Sci.* 119:36–41
52. Ehrlich, H. L. 1976. In *Environmental Biogeochemistry,* ed. J. O. Nriagu, 2:633–44. Ann Arbor, MI: Ann Arbor Sci.
53. Ehrlich, H. L. 1978. *Geomicrobiol. J.* 1:65–83
54. Ehrlich, H. L. 1980. See Ref. 178, pp. 327–32
55. Ehrlich, H. L. 1981. *Geomicrobiology,* pp. 165–259. New York: Dekker. 393 pp.
56. Ehrlich, H. L. 1983. See Ref. 79, pp. 357–66
57. Ehrlich, H. L. 1984. In *Microbial Chemoautotrophy,* ed. W. R. Strohl, O. H. Tuovinen, Ohio St. Univ. Biosci. Colloq. No. 8, Columbus: Ohio St. Univ. Press. In press
58. Eleftheriadis, D. K. 1976. *Mangan- und Eisenoxydation in Mineral- und Thermal-Quellen Mikrobiologie, Chemie und Geochemie.* PhD thesis. Univ. Saarlandes, Sarrbrücken, W. Germany. Cited in Refs. 55, 161
59. Emerson, S., Kalhorn, S., Jacobs, L., Tebo, B. M., Nealson, K. H., Rosson, R. A. 1982. *Geochim. Cosmochim. Acta* 46:1073–79

60. Franklin, F. C. H., Bagdasarian, M., Bagdasarian, M. M., Timmis, K. N. 1981. *Proc. Natl. Acad. Sci. USA* 78: 7458–62
61. Frolund, A. 1977. *Bot. Tidsskrift* 72:45–56
62. Gaudy, E., Wolfe, R. S. 1962. *Appl. Microbiol.* 10:200–5
63. Gebers, R. 1981. *Int. J. Syst. Bacteriol.* 31:302–16
64. Gebers, R., Hirsch, P. 1978. See Ref. 105, pp. 911–21
65. Ghiorse, W. C. 1980. See Ref. 178, pp. 345–54
66. Ghiorse, W. C. 1981. *Abstr. Ann. Meet. Am. Soc. Microbiol.* N64:183
67. Ghiorse, W. C. 1984. In *Current Perspectives in Microbial Ecology*, ed. C. A. Reddy, M. J. Klug. Washington DC: Am. Soc. Microbiol. In press
68. Ghiorse, W. C., Chapnick, S. D. 1983. See Ref. 79, pp. 367–76
69. Ghiorse, W. C., Hirsch, P. 1978. See Ref. 105, pp. 897–909
70. Ghiorse, W. C., Hirsch, P. 1979. *Arch. Microbiol.* 123:213–26
71. Ghiorse, W. C., Hirsch, P. 1982. *Appl. Environ. Microbiol.* 43:1464–72
72. Ghiorse, W. C., Seaman, B., Chapnick, S. D. 1983. *Abstr. 6th Int. Symp. Environ. Biogeochem., Santa Fe, NM*, p. 25
73. Ghiorse, W. C., Wieczorek, L., Hirsch, P. 1980. *Abstr. Ann. Meet. Am. Soc. Microbiol.* N101:180
74. Glathe, H., Ottow, J. C. G. 1972. *Zentralbl. Bakteriol. Parasitenkd. Abt. infektionskr. Hyg. Abt. II. Orig.* 127:749–69
75. Gregory, E., Perry, R. S., Staley, J. T. 1980. *Microb. Ecol.* 6:125–40
76. Gregory, E., Staley, J. T. 1982. *Appl. Environ. Microbiol.* 44:509–11
77. Grosovsky, B. D.-D. *J. Theor. Biol.* 97:83–86
78. Hajj, H., Makemson, J. 1976. *Appl. Environ. Microbiol.* 32:699–702
79. Hallberg, R., ed. 1973. *Environmental Biogeochemistry. Ecol. Bull.* 35:576
80. Hanert, H. 1968. *Arch. Microbiol.* 60:348–76
81. Hanert, H. 1974. *Arch. Microbiol.* 96:59–74
82. Hanert, H. H. 1981. See Ref. 167, pp. 509–15
83. Hanert, H. H. 1981. See Ref. 167, pp. 1049–59
84. Harder, E. C. 1919. *U.S. Geol. Survey Prof. Paper* 113:7–89
85. Harris, R. D., Troup, A. G. 1970. *Limnol. Oceanogr.* 15:702–12
86. Hayat, M. A. 1975. *Positive Staining for Electron Microscopy*, pp. 146–62. New York: Van Nostrand. 361 pp.
87. Heldal, M., Tumyr, O. 1983. *Can. J. Microbiol.* 29:303–8
88. Hirsch, P. 1968. *Arch. Mikrobiol.* 60:201–16
89. Hirsch, P. 1974. *Ann. Rev. Microbiol.* 28:391–44
90. Hoeniger, J. F., Tauschel, H.-D., Stokes, J. L. 1973. *Can. J. Microbiol.* 19:309–13
91. Hogan, V. C. 1973. *Electron Transport and Manganese Oxidation in Leptothrix discophorus*. PhD thesis. Ohio St. Univ. 169 pp.
92. Jackson, D. D. 1901. *Proc. Am. Microsc. Soc.* 23:31–39
93. Jannasch, H. W., Wirsen, C. O. 1981. *Appl. Env. Microbiol.* 41:528–38
94. Jaquet, J.-M., Nembrini, G., Garcia, J., Vernet, J.-P. 1982. *Hydrobiologia* 91:323–40
95. Johnson, A. H., Stokes, J. L. 1966. *J. Bacteriol.* 91:1543–47
96. Jung, W. K., Schweisfurth, R. 1979. *Z. Allg. Mikrobiol.* 19:107–15
96a. Kelly, D. P. 1981. See Ref. 167, pp. 999–1004
97. Kelly, D. P., Norris, P. R., Brierley, C. L. 1979. In *Microbial Technology: Current State, Future Prospects*, 29th Symp. Soc. Gen. Microbiol., ed. A. T. Bull, D. C. Ellwood, C. Ratledge, pp. 263–308. London: Cambridge Univ. Press. 422 pp.
98. Kepkay, P. E. 1983. See Ref. 72, p. 19
99. Kepkay, P. E., Nealson, K. H. 1982. *Abstr. Ann. Meet. Am. Soc. Microbiol.* I110:112
100. Kepkay, P. E., Nealson, K. H. 1982. *J. Bacteriol.* 151:1022–26
101. Kindle, E. M. 1932. *Am. J. Sci.* 24:496–504 5th Ser.
102. Klaveness, D. 1977. *Hydrobiologia* 56: 25–33
103. Konetzka, W. A. 1977. See Ref. 188, pp. 317–42
104. Kono, Y., Fridovich, I. 1983. *J. Biol. Chem.* 258:6015–19
105. Krumbein, W. E., ed. 1978. *Environmental Biogeochemistry and Geomicrobiology: Methods, Metals, and Assessment*, 3:717–1055. Ann Arbor, MI: Ann Arbor Science
105a. Krumbein, W. E., ed. 1983. *Microbial Geochemistry*. Boston: Blackwell Scientific. 330 pp.
106. Krumbein, W. E., Jens, K. 1981. *Oecologia* 50:25–38
107. Kucera, S., Wolfe, R. S. 1957. *J. Bacteriol.* 74:344–49
108. Kuznetsov, S. I. 1970. In *The Microflora of Lakes and Its Geochemical Activity*, ed. C. T. Oppenheimer, pp. 365–407. Austin, TX: Univ. Texas Press. 503 pp.

109. Kuznetsov, S. I., Ivanov, M. V., Lyalikova, N. N. 1962. *Introduction to Geological Microbiology*. Transl. P. T. Broneer, 1969. New York: McGraw-Hill. 252 pp.
110. LaRock, P. A., Ehrlich, H. L. 1975. *Microb. Ecol.* 2:84–96
111. Letunova, S. V., Ulubekova, M. V., Shcherbakov, V. I. 1978. *Microbiology USSR* 47:273–78
112. Lindstrom, M. E., Engebrecht, J., Nealson, K. H. 1983. *FEMS Microbiol. Lett.* 19:1–6
113. Lundgren, D. G., Dean, W. 1979. See Ref. 177, p. 211–51
114. MacRae, I. S., Celo, J. S. 1975. *Appl. Microbiol.* 29:837–40
115. MacRae, I. S., Edwards, J. F. 1972. *Appl. Microbiol.* 24:819–23
116. Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J., McCowen, S. M. 1978. *Plasmid* 1:417–20
117. Mao, M. W. H., Dugan, P. R., Martin, P. A. W., Tuovinin, O. H. 1980. *FEMS Microbiol. Lett.* 8:121–25
118. Mardanyan, S. S., Balashova, V. V. 1971. *Microbiology USSR* 40:104–6
119. Marshall, K. C. 1979. See Ref. 177, pp. 253–92
120. Marshall, K. C. 1980. See Ref. 178, pp. 333–37
121. Martin, P. A. W., Dugan, P. R., Tuovinen, O. H. 1981. *Can. J. Microbiol.* 27:850–53
122. McMillan, L., Stout, R. 1977. *J. Am. Water Works Assoc.* 69:171–73
123. Mills, V. H., Randles, C. I. 1979. *J. Gen. Appl. Microbiol.* 25:205–7
124. Moore, R. L. 1981. *Ann. Rev. Microbiol.* 35:567–94
125. Morgan, J. J., Stumm, W. 1964. *J. Coll. Sci.* 19:347–59
126. Morgan, J. J., Stumm, W. 1965. In *Advances in Water Pollution Research*, ed. O. Jaag, 1:103–18. New York: Pergammon
127. Mulder, E. G. 1964. *J. Appl. Bacteriol.* 27:151–73
128. Mulder, E. G. 1972. *Rev. Ecol. Biol. Sol.* 9:321–48
129. Mulder, E. G., 1974. See Ref. 25, pp. 129–33
130. Mulder, E. G., Deinema, M. H. 1981. See Ref. 167, pp. 425–40
131. Mulder, E. G., vanVeen, W. L. 1963. *Antonie van Leeuwenhoek* 29:121–53
132. Murray, J. W. 1974. *J. Coll. Interface Sci.* 46:357–71
133. Mustoe, G. E. 1981. *Geol. Soc. Am. Bull.* 92:147–53
134. Nealson, K. H. 1978. See Ref. 105, pp. 847–58
135. Nealson, K. H. 1982. In *Mineral Deposits and the Evolution of the Biosphere. Dahlem Konferenze*, ed. H. D. Holland, M. Schidlowski, pp. 51–66. New York: Springer. 332 pp.
135a. Nealson, K. H. 1983. See Ref. 105a, pp. 159–190
135b. Nealson, K. H. 1983. See Ref. 105a, pp. 191–221
136. Nealson, K. H., Tebo, B. 1970. *Orig. Life* 10:117–26
137. Nealson, K. H., Ford, J. 1980. *Geomicrobiol. J.* 2:21–37
138. Nunley, J. W., Kreig, N. R. 1968. *Can. J. Microbiol.* 14:385–89
139. Palleroni, N. J. 1983. *Bioscience* 33: 370–77
140. Perfil'ev, B. V., Gabe, D. R. 1961. *Capillary Methods of Investigating Microorganisms*. Transl. J. Shewan, 1969. Edinburg: Oliver & Boyd. 627 pp.
141. Petitprez, M., Leclerc, H. 1969. *Ann. Inst. Pasteur-Lille* 20:115–40
142. Petitprez, M., Petitprez, A., Leclerc, H., Vivier, E. 1969. *Ann. Inst. Pasteur-Lille* 20:103–14
143. Pringsheim, E. G. 1949. *Biol. Rev.* 24:200–45
144. Pringsheim, E. G. 1949. *Philos. Trans. R. Soc. London Ser. B* 233:453–82
145. Pringsheim, E. G. 1952. *Endeavour* 11:208–14
146. Puchelt, H., Schock, H. H., Schroll, E., Hanert, H. 1973. *Geol. Rundschau* 62:786–812
147. Ridgway, H. F., Means, E. G., Olson, B. H. 1981. *Appl. Environ. Microbiol.* 41:288–97
148. Ridgway, H. F., Olson, B. H. 1981. *Appl. Environ. Microbiol.* 41:247–87
149. Rogers, S. R., Anderson, J. J. 1976. *J. Bacteriol.* 126:257–63
150. Rogers, S. R., Anderson, J. J. 1976. *J. Bacteriol.* 126:264–71
151. Romano, A. H., Geason, D. J. 1964. *J. Bacteriol.* 88:1145–50
152. Romano, A. H., Peloquin, J. P. 1963. *J. Bacteriol.* 86:252–58
153. Rosson, R. A., Nealson, K. H. 1982. *J. Bacteriol.* 151:1027–34
154. Rosswall, T., ed. 1973. *Modern Methods in the Study of Microbial Ecology, Ecol. Bull.*, Vol. 17. 511 pp.
155. Rouf, M. A., Stokes, J. L. 1964. *Arch. Mikrobiol.* 49:132–49
156. Savel'eva, N. D. 1965. *Microbiology USSR* 34:780–84
157. Schmidt, J. M., Sharp, W. P., Starr, M. P. 1982. *Curr. Microbiol.* 7:389–94
158. Schmidt, W.-D. 1979. *Morphologie und Physiologie Manganoxidierender Mikroorganismen-Kultur-und* in situ *untersuchungen zur ökologisch-mikrobiologischen Charakterisierung von* Met

allogenium sp. *und* Siderocapsa geminata *im Plusssee*. PhD thesis. Univ. Kiel, W. Germany. 215 pp.
159. Schütt, C., Ottow, J. C. G. 1978. See Ref. 105, pp. 869–78
160. Schweisfurth, R. 1973. *Z. Allg. Mikrobiol.* 13:341–47
161. Schweisfurth, R., Eleftheriadis, D., Gundlach, H., Jacobs, M., Jung, W. 1978. See Ref. 105, pp. 923–28
162. Schweisfurth, R., Hehn, G. V. 1972. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. A* 220:357–61
163. Siebke, R. 1979. *Untersuchung der Feinstruktur von Eisenund/oder Mangan-Ablagernden Mikroorganismen*. Diplomarbeit thesis. Univ. Kiel, W. Germany. 91 pp.
164. Silver, S., Jasper, P. 1977. See Ref. 188, pp. 105–49
165. Smith, S. 1982. *Ground Water* 20:482–85
166. Starkey, R. L. 1945. *J. Am. Water Works Assoc.* 37:963–84
167. Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., Schlegel, H. C., eds., 1981. *The Prokaryotes. A Handbook on Habitats, Isolation, and Identification of Bacteria*, Vol. 1. New York: Springer. 1022 pp.
168. Stokes, J. L. 1954. *J. Bacteriol.* 67:278–91
169. Stokes, J. L., Powers, M. T. 1967. *Arch. Microbiol.* 59:295–301
170. Stumm, W., Morgan, J. J. 1981. *Aquatic Chemistry. An Introduction Emphasizing Chemical Equilibria in Natural Waters*, pp. 418–99. New York: Wiley. 780 pp.
171. Summers, A. O., Silver, S. 1978. *Ann. Rev. Microbiol.* 32:637–72
172. Svorcova, L. 1983. *Acta Hydrochim. Hydrobiol.* 11:37–45
173. Taylor-George, S., Palmer, F., Staley, J. T., Borns, D. J., Curtiss, B. et al. 1983. *Microbial Ecol.* 9:227–45
174. Tebo, B. M. 1983. *Ecology and ultrastructure of marine manganese oxidizing bacteria*. PhD thesis. Univ. Calif., San Diego. 220 pp.
175. Temple, K. L., Colmer, A. R. 1951. *J. Bacteriol.* 62:605–11
176. Timonin, M. T. 1950. *Sci. Agr.* 30:324–25
177. Trudinger, P. A., Swaine, D. J., eds. 1979. *Biogeochemical Cycling of Mineral-Forming Elements*. New York: Elsevier. 612 pp.
178. Trudinger, P. A., Walter, M. R., Ralph, B. J., eds. 1980. *Biogeochemistry of Ancient and Modern Environments*. New York: Springer. 723 pp.
179. Tuovinen, O. H., Nurmiaho, E.-L. 1979. *Microb. Ecol.* 5:57–66
180. Tyler, P. A. 1970. *Antonie van Leeuwenhoek* 36:567–78
181. Tyler, P. A., Marshall, K. C. 1967. *Antonie van Leeuwenhoek* 33:171–83
182. Tyler, P. A., Marshall, K. C. 1967. *Arch. Mikrobiol.* 56:344–53
183. Tyler, P. A., Marshall, K. C. 1967. *J. Am. Water Works Assoc.* 59:1043–48
184. vanVeen, W. L. 1973. *Antonie van Leeuwenhoek* 39:657–62
185. vanVeen, W. L., Mulder, E. G., Deinema, M. H. 1978. *Microbiol. Rev.* 42: 329–56
186. Vatter, A. E., Wolfe, R. S. 1956. *J. Bacteriol.* 72:248–52
187. Völker, H., Schweisfurth, R., Hirsch, P. 1977. *J. Bacteriol.* 131:306–13
188. Weinberg, E. D., ed. 1977. *Microorganisms and Minerals*. New York: Dekker. 492 pp.
189. Wieczorek, L., Ghiorse, W. C., Hirsch, P. 1980. *Abstr. Ann. Meet. Am. Soc. Microbiol.* N102:180
190. Winogradsky, S. 1888. *Bot. Ztg.* 46: 262–70
191. Wolfe, R. S. 1958. *J. Am. Water Works Assoc.* 50:1241–49
192. Wolfe, R. S. 1960. *J. Am. Water Works Assoc.* 52:915–18
193. Wolfe, R. S. 1960. *J. Am. Water Works Assoc.* 52:1335–39
194. Wolfe, R. S. 1964. In *Principles and Applications and Aquatic Microbiology*, ed. H. Heukelekian, N. C. Dondero, pp. 82–97. New York: Wiley. 452 pp.
195. Zapkin, M. A., Ehrlich, H. L. 1983. *Z. Allg. Mikrobiol.* 23:447–55
196. Zavarzin, G. A. 1968. In *The Ecology of Soil Bacteria*, ed. T. R. G. Gray, D. Parkinson, pp. 612–23. Liverpool Univ. Press. 681 pp.
197. Zavarzin, G. A. 1981. See Ref. 167, pp. 524–28
198. Zavarzin, G. A., Hirsch, P. 1974. See Ref. 25, pp. 160–61
199. Zhdanov, A. V., Dubinina, G. A. 1975. *Microbiology USSR* 44:644–49
200. Zindulis, J., Ehrlich, H. L. 1983. *Z. Allg. Mikrobiol.* 7:457–65

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HYDROGENASE, ELECTRON-TRANSFER PROTEINS, AND ENERGY COUPLING IN THE SULFATE-REDUCING BACTERIA *DESULFOVIBRIO*

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INTRODUCTION

Until the late 1970s, the sulfate-reducing bacteria (SRB) were regarded as a small, specialized group of strictly anaerobic bacteria with the limited metabolic capability of oxidizing short-chain organic acids to acetate with the concomitant reduction of sulfate to sulfide (155). The SRB were classified into two genera, *Desulfovibrio* and *Desulfotomaculum*, in the mid-1960s (43, 156), and it was assumed that the mechanism and bioenergetics of respiratory sulfate reduction was identical in the two genera. Biochemically, the genus *Desulfovibrio* was characterized as containing *c*-type cytochromes and producing an intense red fluorescence in the presence of alkali and ultraviolet light (365 nm), which was due to green chromatophore desulfoviridin (154)—later shown to be a bisulfite reductase (107, 109). The genus *Desulfotomaculum* characteristically lacks *c*-type cytochromes and desulfoviridin but forms spores.

The recent description of six new genera of SRB [*Desulfobacter* (196), *Desulfosarcina* (195), *Desulfonema* (151, 198), *Desulfobulbus* (197), *Desulfococcus* (151, 195) and *Thermodesulfobacterium* (212)], which are biochemically, nutritionally, and morphologically diverse, has completely changed the concept that the SRB are a small, specialized group of bacteria with limited metabolic capabilities. The different genera of SRB have only in common their ability to utilize sulfate as a terminal electron acceptor and the fact that they are all strict anaerobes. On the basis of the isolation of these new types of SRB and a fuller appreciation of the ecology of the SRB in anaerobic environments, it has been suggested (151) that the SRB be taxonomically considered as one physiological-ecological group, as has been done with the methanogenic and phototrophic bacteria. Thus, it is to be expected that the known types of SRB will continue to be expanded, and additional physiological and biochemical diversity will be found within this broad and important group of microorganisms.

The genus *Desulfovibrio* has been the most extensively studied of the SRB (115, 155). Their bioenergetics at the physiological and molecular levels will be the focus of this review. Members of the genus *Desulfovibrio* do not constitute a uniform group and exhibit many differences that cannot be regarded as trivial. The intense red fluorescence produced in the presence of alkali under ultraviolet light has been considered as definitive taxonomic characteristic of this genus; however, three species of *Desulfovibrio* have now been reported that do not exhibit this characteristic: *D. baculatus* (159), *D. sapovorans* (151), and *D. desulfuricans* (Norway 4) (130). In the Norway

strain, desulfoviridin is replaced by a different bisulfite reductase, desulforubidin, a bisulfite reductase that releases nonfluorescent siroheme rather than siroporphyrin in the presence of alkali (108, 137). It should also be noted that desulfoviridin has been detected in extracts of *Desulfococcus multivorans* and *Desulfonema limicola* (195), and desulforubidin has been detected in extracts of *Desulfobacter postgatei* (C. L. Liu & H. D. Peck, Jr., unpublished data). The presence of desulfoviridin can thus no longer be regarded as a taxonomic characteristic of the genus *Desulfovibrio*.

The removal of acetate from anaerobic environments was believed to be solely due to the metabolic activities of the methanogenic bacteria until Pfennig & Biebl (150) isolated a sulfur-reducing bacterium, *Desulfuromonas*, capable of catalyzing the complete oxidation of acetate to CO_2 in the presence of elemental sulfur. This most important observation has now been extended to include some members of the SRB (151); they can be divided into two distinctive groups depending on whether or not acetate and/or propionate is accumulated during growth with organic substrates plus sulfate. Extracts of *Desulfobacter postgatei* grown on acetate and sulfate were found to contain most of the enzymes of the citric acid cycle, which has been proposed as the pathway for the anaerobic oxidation of acetate with sulfate (176). Substrate level phosphorylation cannot provide the ATP required for sulfate reduction and growth; thus another mechanism of energy conservation must be linked to the anaerobic oxidation of acetate by these bacteria. The presence or absence of the ability to oxidize acetate appears to be a major physiological character among the SRB. The physiological diversity of members of *Desulfovibrio* is further indicated by the presence of both acetate-oxidizing and acetate-accumulating species in the genus (150, 151).

The metabolism of hydrogen has played a central role in the development of our concepts concerning the SRB; however, there exists considerable diversity at both the physiological and biochemical levels. Although all members of *Desulfovibrio* studied exhibit high levels of hydrogenase activity, variable amounts of H_2 are accumulated during the lactate-sulfate fermentation (71, 178). Two distinct and well-characterized periplasmic hydrogenases have been demonstrated in different species of *Desulfovibrio:* a nickel-containing nonheme iron protein, (NiFe) hydrogenase, (41, 118) and a nonheme iron protein, (Fe) hydrogenase (80). The two hydrogenases can be differentiated on the basis of metal content, specific activity, subunit structure, reactivity towards inhibitors (see Table 2), and, apparently, antigenicity (8). The latter observations are particularly interesting, because they suggest that even different strains of *D. vulgaris* can differ with regard to the type of hydrogenase produced.

At the molecular level, the interspecies heterogeneity of the tetraheme protein cytochrome c_3 (M_r = 13,000), which is unique to *Desulfovibrio*, has

been extensively documented in several laboratories (116, 211). Seven amino acid sequences of cytochrome c_3 from four different species of *Desulfovibrio* and different strains of *D. vulgaris* and *D. desulfuricans* have been determined. Surprisingly, homology can be as low as 25% between cytochromes of different species; within one species, *D. vulgaris* (Hildenborough) and *D. vulgaris* (Miyazaki) differ by 14 amino acid residues out of a total of 107 residues. For comparison, it should be noted that cytochrome *c* from man and from pigeon differ by 12 out of a total of 104 amino acid residues. These observations demonstrate that considerable modification of individual proteins has occurred during evolution within this genus of anaerobic microorganisms. As yet unanswered are questions regarding the impact of this diversity on the complex activities of electron transfer and energy coupling.

ELECTRON TRANSFER PROTEINS—*AN OVERVIEW*

Cytochrome c_3 was the first *c*-type cytochrome to be discovered in a nonphotosynthetic strict anaerobe as well as the first electron-transfer protein to be isolated from *Desulfovibrio* (82, 153). Subsequently, ferredoxin, flavodoxin, rubredoxin, and a monoheme cytochrome c_{553} were demonstrated in the SRB. These initial observations on electron-transfer proteins have been extended to many species of *Desulfovibrio,* and additional types of electron-transfer proteins and redox protein have been found (116). The distribution of selected electron-transfer components in *Desulfovibrio* is shown in Table 1. Cytochrome c_3 (M_r = 13,000) and cytochrome c_3 (M_r = 26,000) are found in all species of *Desulfovibrio* examined and represent a unique class of cytochromes (88); however, flavodoxin, ferredoxin, and cytochrome c_{553} are present in only trace amounts or absent in some species of *Desulfovibrio*.

The original and continuing goals of these intensive studies of the electron-transfer proteins of *Desulfovibrio* have been four-fold: first, to expand our knowledge concerning the biological diversity of redox proteins; second, to utilize the natural variability of these electron-transfer proteins to investigate structure/function relationships of redox proteins; third, to understand the nature of electron transfer in the SRB; and fourth, to utilize biochemical information concerning these electron-transfer proteins to probe the mechanisms of energy coupling involved in respiratory sulfate reduction. These are clearly long range goals, but significant recent progress has been made in each area. At least three new types of low-molecular-weight redox proteins unique to *Desulfovibrio* have been described: cytochrome c_3 (M_r = 26,000) (34), desulforedoxin (131), and the cobalt porphyrin–containing protein (68, 134). Oligomeric forms of ferredoxin have been described that can contain either a four-iron/four-sulfur or a three-iron/three-sulfur cluster (117). The tetraheme

Table 1 Distribution of hydrogenase and electron-transfer proteins in various *Desulfovibrio*

Organism	Hydrogenase*		Cytochromes			Electron Transfer Proteins				
						Ferredoxins				
	(Fe)	(NiFe)	C_{553} or monoheme	c_3 (13,000)	c_3 (26,000)	$[Fe_4S_4]$	$2[Fe_4S_4]$	$[Fe_3S_4]$	Fl	Rb
D. vulgaris (Hildenborough)	+	–	+	+	+	1	NR	NR	+	+
D. gigas	–	+	–	+	+	+	–	+	+	+
D. desulfuricans (Norway 4)	–	+	+	+	+	1	1	NR	–	+
D. desulfuricans (ATCC 27774)	–	+	–	+	+	NR	NR	NR	+	+
D. africanus	NR	NR	NR	+	+	2	1	NR	–	+

*+ = present; – = absent; NR = not reported; Fl = flavodoxin; Rb = rubredoxin.

nature of cytochrome c_3 has been conclusively established (12, 129, 208), and electron transfer between its four hemes extensively studied (135, 162, 188). Flavodoxin from *D. vulgaris* provided the material for the first three-dimensional structure of a flavoprotein (193), and most recently nickel has been shown to be a redox-active component of the hydrogenases from *D. gigas* and *D. desulfuricans*.

Studies on the structure of the electron-transfer components are far more advanced than studies on the function. This is due in part to the fact that some electron-transfer proteins exhibit an apparent lack of specificity (i.e. in most reactions flavodoxin can substitute for ferredoxin) and in part to the fact that many of these proteins appear to be compartmentalized. Thus, when extracts are prepared, enzymes and electron-transfer proteins from the periplasm, membranes, and cytoplasm are mixed, and physiological specificity afforded by their localization is lost.

Several unique and important observations concerning the specificities of cytochrome c_3 (M_r = 13,000) and ferredoxins have been made. Cytochrome c_3 has been demonstrated to act as a electron carrier for both the (Fe) hydrogenase of *D. vulgaris* and the (NiFe) hydrogenase of *D. gigas;* this appears to be its main function in *Desulfovibrio* (27, 206). The oligomeric forms of ferredoxin from *D. gigas* exhibit an extremely interesting type of specificity: the ferredoxin containing the three-iron cluster is only active in the reduction of sulfite with hydrogen, whereas the ferredoxin containing the four-iron cluster is only active in the phosphoroclastic reaction (133). The observations suggest that specific reactions for other electron-transfer proteins may be anticipated.

Investigations into the amino acid sequences of electron-transfer proteins involved in electron transfer has not been highly productive. This has been due to different specificities of electron carriers from different species of *Desulfovibrio* (38) and lack of definitive experiments regarding the pathway of bisulfite reduction (6). The importance of cellular localization (18, 148) and diversity of electron-transfer proteins has only recently been appreciated and should promote future progress in this area. The use of biochemical information to study the mechanisms of energy coupling will be discussed in the next section.

The electron-transfer proteins from *Desulfovibrio* have been more extensively studied than similar proteins from any other genus of microorganisms. A large amount of biochemical data concerning these proteins is available; however, the actual function of many of the individual electron-transfer proteins remains obscure. Clearly, the mechanisms of electron transfer and energy coupling in these microorganisms are not "primitive" as previously postulated (146), and the individual classes of protein exhibit an unexpectedly high degree of variability within the genus, *Desulfovibrio,* that may reflect changes in function in different species.

ENERGY COUPLING—*AN OVERVIEW*

The first demonstration of electron transfer–coupled phosphorylation in non-photosynthetic anaerobic bacteria was made with SRB belonging to the genus *Desulfovibrio* (144). The oxidation of hydrogen with sulfate by intact cells was shown to be reversibly inhibited by 2,4-dinitrophenol, whereas the oxidation of hydrogen with sulfite or thiosulfate was not inhibited. The explanation presented for this selective inhibition was based on the fact that ATP is required only for the reduction of sulfate and that 2,4-dinitrophenol uncouples ATP production by electron transfer–coupled phosphorylation. Results with cell-free preparations and growth yields also support these initial observations. Cell-free preparations of *D. gigas* can couple the synthesis of ATP to the oxidation of hydrogen with sulfite (147), fumarate (22, 23) and sulfur (56). These systems exhibit low P/H_2 ratios (147) but are sensitive to uncoupling agents and do not require pyridine nucleotides for electron transfer.

The bioenergetics of *Desulfovibrio* has also been studied by means of growth yields with various electron donors and acceptors (124, 163, 190); however, the growth characteristics of the bacteria did not allow definitive interpretations of the data. More recently, several strains of *Desulfovibrio* that can grow on hydrogen, sulfate, carbon dioxide, and acetate have been described (17, 31); they conclusively demonstrate that a net formation of ATP is coupled to the oxidation of hydrogen with sulfate. Analysis of the growth-yield data with sulfate and thiosulfate as terminal electron acceptors for the oxidation of hydrogen indicate that the formation of one net ATP is coupled to the reduction of each sulfate and three net ATPs to the reduction of each thiosulfate (regarded as the equivalent of sulfite). These three different types of observations clearly indicate that in *Desulfovibrio,* electron transfer–linked phosphorylation is coupled to the oxidation of hydrogen and, by inference, to the anaerobic oxidation of organic substrates (145).

Evidence is accumulating that there exist two chemiosmotic mechanisms for the generation of proton gradients in *Desulfovibrio* and possibly in other microorganisms (149). One mechanism involves proton translocation during electron transfer via a Mitchell-type loop or specific enzymes. A second mechanism consists of the scalar production of a protons as a result of the oxidation of an electron donor (such as hydrogen or formate) on the external surface of the cytoplasmic membrane coupled by electron transfer across the membrane and the cytoplasmic reduction of an electron acceptor. The existence of the first mechanism is indicated by the observation that during the reduction of nitrite with hydrogen, a proton gradient is generated that cannot be accounted for by the scalar production of protons (169). It would also be necessary to invoke this mechanism to explain electron-transfer phosphorylation observed between redox couples, such as lactate and fumarate, whose

primary dehydrogenases are localized in the cytoplasm or internal aspect of the cytoplasmic membrane (L. L. Barton & H. D. Peck, Jr., unpublished data).

Experimental evidence for the second mechanism was first presented by Kroger (99, 100) in studies on the bioenergetics of *Wolinella succinogenes* (formerly *Vibrio succinogenes*) (174) growing with formate and fumarate. He proposed that a proton gradient is generated across the cytoplasmic membrane by the periplasmic (external) oxidation of formate to CO_2 and protons. Electrons only are transferred across the membrane and coupled to the cytoplasmic reduction of fumarate to succinate by a membrane-bound fumarate reductase (101). The net effect is the generation of a proton gradient as a result of the production of protons in the periplasm, owing to formate oxidation and the consumption of protons in the cytoplasm owing to fumarate reduction. The process has been termed vectorial electron transfer and has an $H^+/2e$ ratio of 2.0 that is in agreement with growth-yield studies (100), assuming $2H^+$ per ATP formed. The generation of ATP is coupled to the oxidation of hydrogen with fumarate (157), and it has been suggested that this phenomenon is also due to the scalar production of protons resulting from the periplasmic oxidation of hydrogen (102).

A similar mechanism has been proposed for energy coupling during the oxidation of hydrogen with sulfate by the Marburg strain of *D. vulgaris* (18). As with *W. succinogenes*, the oxidation of hydrogen by the periplasmic hydrogenase results in the generation of a proton gradient that supports ATP synthesis by means of a reversible ATPase (60). Electrons transferred across the membrane would be utilized for the cytoplasmic reduction of sulfate to sulfide with concomitant consumption of protons. The topology of the enzymes and electron-transfer proteins is consistent with this proposal. This mechanism does not require the direct coupling of proton translocation to electron transfer as proposed by Wood (204) for dissimilatory sulfate reduction and represents a simple chemiosmotic mechanism for energy coupling.

Based on these results and concepts concerning the conservation of hydrogen produced during the fixation of nitrogen (49), we have proposed a bioenergetic mechanism for the growth of *Desulfovibrio* on organic substrates plus sulfate (139) that requires the cytoplasmic production of molecular hydrogen from organic substrates, diffusion across the cytoplasmic membrane, and oxidation of hydrogen by the periplasmic hydrogenase coupled to vectorial electron transfer to sulfate. The concept of hydrogen cycling for energy coupling offers a framework within which a number of unique physiological, enzymological, and biochemical observations concerning dissimilatory sulfate reduction can be explained: for example, the unique ability of *Desulfovibrio* to grow in consortia by interspecies H_2 transfer as either the hydrogen producing- (39) or hydrogen-utilizing bacterium (30). Mechanistically, it is relatively simple and may represent a stage in the evolution of proton translocation. Clearly, hydrogen

cycling should be investigated in additional species of *Desulfovibrio* and in other types of anaerobic bacteria, including the new genera of sulfate-reducing bacteria.

HYDROGENASES

Desulfovibrio gigas

The hydrogenase activity of *D. gigas* largely resides in the periplasm (26); however, a fraction of the activity appears to be cytoplasmic after the formation of spheroplasts (139). The enzyme responsible for the cytoplasmic hydrogenase activity has not been identified, but the periplasmic hydrogenase has been well characterized. It is a nonheme iron protein with a M_r of 89,500 and is composed of nonidentical subunits of 62,000 and 26,000 daltons (72). The hydrogenase contains 11–12 g atom of nonheme iron and sulfide per molecule, and by chemical determination (41, 118) and isotopic substitution (136) was shown to contain 1 g atom of nickel per molecule. Specific activity of the hydrogenase in the evolution assay is 440μ mol H_2/min/mg protein (118). In the oxidized state at 10°K, the hydrogenase exhibits an isotropic EPR signal at $g = 2.02$ superimposed on rhombic EPR signals with g values at 2.31, 2.23, and 2.02 (41, 118). In conjunction with Mössbauer spectroscopy, the isotropic signal has been assigned to a three-iron cluster, and two "EPR silent" (Fe_4S_4) clusters were detected (136). Nickel can commonly exist in three redox states, Ni(I), Ni(II), and Ni(III), but only Ni(I) and Ni(III) are paramagnetic and can be detected by EPR spectroscopy (106).

The rhombic EPR signals have been assigned to nickel (III) based on the following observations: first, the EPR signals can be observed up to 120°K; second, nickel-61 induces hyperfine structure in the EPR spectra; and third, the observed g values are similar to those for Ni(III) in other biological materials and model compounds. Reduction of the hydrogenase either with hydrogen or with limiting amounts of dithionite results in the appearance of a new set of EPR signals with major g values at 2.19 and 2.13 that disappear in the presence of excess dithionite (118). The E_o' values for the nickel and three-iron centers have been determined as −220 mV and −70 mV respectively, and the redox potential of the nickel signal was shown to be pH-dependent (41, 175). In the presence of dithionite, the redox centers are reduced in the order of their redox potentials, i.e. the three-iron cluster first and then the nickel center; however, in the presence of hydrogen the order is reversed. The data have been interpreted as indicating that nickel is involved in the activation of H_2 and undergoes a Ni(III) $\rightleftharpoons$ Ni(II) redox transition (41, 175).

The role of the three-iron cluster has not yet been ascertained: its redox potential is too high to be involved in the activation of hydrogen; however, it may be a regulatory center and converted to a (Fe_4S_4) center on reduction of the

enzyme, as occurs with aconitase (92). Although such a conversion has been described for a ferredoxin from *D. gigas* (91), the conversion of the three-iron center of hydrogenase has not yet been reported. More information regarding the liganding of the nickel and the redox properties of the "EPR silent" (Fe_4S_4) centers are required before a mechanism for the activation of hydrogen can be proposed.

Oxygen can effect irreversible and reversible inactivations of hydrogenases (3). Examples of the irreversible inactivation by oxygen are the hydrogenases from *C. pasteurianum* and *M. elsdenii* (44, 186), which must be purified in the complete absence of air. Most of the so-called "oxygen-stable" hydrogenases such as those found in the SRB are reversibly inactivated by oxygen and are only fully active under anaerobic conditions after exposure to activators such as hydrogen, dithionite, reduced benzyl viologen, or cytochrome c_3 (M_r = 13,000). The function of the activators is probably to remove oxygen that is bound to an essential redox center. The hydrogenase of *D. gigas* as isolated is inactive in the deuterium-exchange assay but can be activated by dithionite, cytochrome c_3, or long exposure to hydrogen (28). The hydrogenase from *D. gigas* requires the presence of cytochrome c_3 (M_r = 13,000) for the reduction of ferredoxin, rubredoxin, cytochrome c_3 (M_r = 26,000) with hydrogen and for the evolution of hydrogen from dithionite (27). The requirement of the cytochrome for the reduction of electron-transfer components might be explained as activation; however, its requirement for the evolution of hydrogen from dithionite conclusively demonstrates that electron transfer between hydrogenase and cytochrome c_3 occurs.

CO has been frequently studied as a general inhibitor of hydrogenase activity. The hydrogenases from the SRB that have been studied are sensitive to CO inhibition and the inhibition is completely reversible. The (NiFe) hydrogenases are much less sensitive to CO than the (Fe) hydrogenase. Thus, the K_i for CO of the hydrogenase from *D. gigas* is 100-fold higher than the K_i for the hydrogenase from *D. vulgaris* (Y. Berlier, G. Fauque, J. LeGall, P. A. Lespinat & H. D. Peck, Jr., unpublished data). This is a significant difference between the two types of hydrogenase, which may be related to their metal content.

D. vulgaris (Hildenborough)

Before discussing the hydrogenase of *D. vulgaris*, two taxonomic considerations need to be mentioned. Prior to the reclassification in 1966 (156), the most commonly studied SRB, *D. vulgaris* (Hildenborough), (NCIB 8303) was classified as *D. desulfuricans*. More recently, *D. vulgaris* (Miyazaki), which has been used by Japanese scientists for their extensive studies on the biochemistry of dissimilatory sulfate reduction, was found to include three strains: MK, MF, and MY. Although all three strains were identified as *D. vulgaris*,

significant differences do exist among the various Miyazaki strains (8, 97, 211). Because many of their papers were published without sufficient strain identification information, they cannot be discussed in detail within the context of this review.

The first attempt at the purification of hydrogenase from *D. vulgaris* (NCIB 8303) was reported by Sadana & Jagannathan (160), and, because of its stability and high specific activity, it was subsequently studied in a number of laboratories (65, 114, 161, 209). Pure hydrogenase from *D. vulgaris* was first obtained by selective elution of the periplasmic proteins, which contained over 90% of the hydrogenase activity (183). The hydrogenase has a M_r of 50,000, consists of a single polypeptide chain, and contains 12.5 g atoms nonheme iron and 11.5 g atom of acid-labile sulfide per mol, arranged in at least two (Fe_4S_4) clusters of the ferredoxin type. The hydrogenase has a specific activity (μmol H_2/min/mg protein) of 4600 in the hydrogen-evolution assay with methyl viologen and 50,000 in the hydrogen-consumption assay with benzyl viologen (127). Long exposure of the oxidized enzyme to oxygen does not irreversibly inactivate the hydrogenase; however, in the reduced form, the hydrogenase is irreversibly inactivated by oxygen (184).

Purified hydrogenase from *D. vulgaris* has been reported (59) to contain variable amounts of nickel, ranging from 0.1–0.6 g atoms per mol of hydrogenase, but the presence of an EPR signal due to redox-active nickel has not been detected. Hydrogenase from this bacterium has been prepared that does not contain significant amounts of nickel, and on the basis of this observation it has been concluded that the hydrogenase from *D. vulgaris* does not contain nickel as an essential constituent (80). The enzyme as isolated shows a small isotropic signal at $g = 2.02$ (0.05–0.15 spin per molecule of hydrogenase), which is typical of a three-iron cluster; however, it was not possible to detect the presence of such a cluster by Mössbauer spectroscopy. These data indicate that either the hydrogenase does not contain significant amounts of the three-iron cluster or that the isotropic EPR signal has a different origin. The hydrogen-reduced hydrogenase exhibits, below 20°K, a typical ferredoxin-type g = 1.94 EPR signal that accounts for 1.4 spins per molecule. An axial EPR signal with a g value of 2.06 is also observed that can be observed at 77°K and is enhanced 10-fold (0.3 spins per molecule) by the addition of mild oxidizing agents such as 2,6-dichlorophenondophenol or $Fe(CN)_6^{-3}$ (185). Iron-57–enriched hydrogenase was prepared, and its EPR spectrum shows a broadened $g = 2.06$ signal that unequivocally demonstrates this EPR signal is derived from an iron-containing complex rather than a complex that contains nickel or another metal (80). The $g = 2.06$ signal has also been observed in the presence of $Fe(CN)_6^{-3}$; however, exposure to this oxidizing agent resulted in loss of activity, and the $g = 2.06$ signal was attributed to a damaged (Fe_4S_4) cluster (59). The presence of this paramagnetic nonheme iron cluster has also been confirmed by Mössbauer spectroscopy (80).

The purified hydrogenase from *D. vulgaris* reduces cytochrome c_3 (M_r = 13,000) (206) but not cytochrome c_{553}. The presence of cytochrome c_3 does not inhibit or stimulate activity as has been reported for the hydrogenase from *D. vulgaris* (Miyazaki) (210). In the deuterium exchange reaction, cytochrome c_3 causes an activation of the hydrogenase but does not effect the overall rate of exchange. When cytochrome c_3 is added to the hydrogen-reduced hydrogenase (80), an increase in the g = 2.06 EPR signal occurs comparable to that observed with other oxidizing agents. Because of the fact that cytochrome c_3 has no effect on the catalytic activity of hydrogenase (187a), the g = 2.06 signal cannot be attributed to an inactivated nonheme iron cluster (185); rather it appears to be a novel type of nonheme iron cluster that may be of importance for the catalytic activity of the hydrogenase of *D. vulgaris*.

As with other hydrogenases, the enzyme from *D. vulgaris* is reversibly inhibited by carbon monoxide. In the deuterium exchange, 50% inhibition occurs at 0.1 μM, and the inhibition is completely reversible (Y. Berlier, G. Fauque, J. LeGall, P. A. Lespinat & H. D. Peck, Jr., unpublished data). Reaction of the hydrogen-reduced hydrogenase with CO results in an increase in the g = 2.06 EPR that may be due to spin decoupling (B. H. Huynh, personal communication) because hydrogenase has not been reported to catalyze the oxidation of CO to CO_2. In addition, by Mössbauer spectroscopy, a new component is observed with a large magnetic splitting unusual for a nonheme iron cluster (80). Because this atypical cluster only appears in CO-reacted hydrogenase, it is likely that the new component represents a CO-bound iron cluster. It has not yet been determined whether the formation of this new component is reversible.

Antibodies to the hydrogenase of *D. vulgaris* that inhibit its catalytic activity have been produced in several laboratories. The antibodies inhibit one of the two types of hydrogenase produced by *M. elsdenii* but do not inhibit the hydrogenases of *D. gigas* and several other species of SRB (8, 187). This data also supports the conclusion that the periplasmic hydrogenases from *D. vulgaris* and *D. gigas* are completely different enzymes.

D. desulfuricans

The first strain of *D. desulfuricans* to be discussed was isolated in the laboratory of M. P. Bryant, contains desulfoviridin, and is identified by its American Type Culture Collection Number, 27774. It was selected for study because of its ability for utilizing nitrate, in addition to sulfate, as its terminal electron acceptor, thus avoiding the formation of ferrous sulfide in studies with iron-57. A portion of the hydrogenase activity is localized on the external aspect of the cytoplasmic membrane (169), but conditions for its selective elution have not been established. Hydrogenase activities were therefore purified from a total extract (French press), and two protein fractions exhibiting hydrogenase activity were isolated (103). Hydrogenase I had a M_r of 77,600, and hydrogenase II had a M_r of 75,500; both enzymes were composed of two subunits and contained nickel and nonheme iron.

These observations are similar to the report that two hydrogenases, 47,000 and 49,000 daltons, can be purified from total extracts of *M. elsdenii* (187). Because of its higher specific activity, hydrogenase II was more intensively studied, but both appeared to have identical redox centers. The EPR spectrum of the purified hydrogenase II shows an isotropic signal at g = 2.02 and a rhombic signal with g values at 2.32, 2.21, and 2.01. At temperatures higher than 77°K, the g = 2.02 signal can no longer be observed, but the rhombic-type signals remain. These results are similar but not completely identical to the results obtained with the hydrogenase from *D. gigas*. Hydrogenase II enriched in nickel-61 and iron-57 was also isolated, and the hyperfine patterns of the EPR spectra allowed the assignment of the g = 2.02 signal to iron and the rhombic signals to nickel. Upon reduction with hydrogen, the oxidized signals disappear and are replaced by a strong g = 1.94 signal typical of ferredoxin-type (Fe_4S_4) cluster and weak signals probably due to nickel. Mössbauer studies of the iron-57 hydrogenase demonstrated the presence of one three-iron cluster and two (Fe_4S_4) clusters. In general, the EPR and Mössbauer results obtained with the hydrogenase of *D. desulfuricans* are comparable to the results obtained with the hydrogenase of *D. gigas*, except for the g = 1.94 signal always observed in the reduced state.

Neither the electron-carrier specificity or the effect of inhibitors has been studied with this hydrogenase. Hydrogenase has also been isolated from a second strain of *D. desulfuricans* (NRC 49001) that contains desulfoviridin. It was purified by selective elution of the periplasmic proteins and consists of a single polypeptide chain of M_r 52,000 (58). The enzyme is oxygen stable and contains 12 nonheme irons and labile sulfide. It has high activity in the evolution assay (however, note low activity in consumption assay, Table 2) and, although information on the nonheme iron centers was not reported, it appears to be similar to the (Fe) hydrogenase of *D. vulgaris*.

D. desulfuricans (Norway 4)

The hydrogenase from the Norway 4 strain of *D. desulfuricans* is of interest, because the bisulfite reductase is desulforubidin rather than desulfoviridin (108). The membrane-bound hydrogenase has been purified and characterized, but it has not been determined whether it is localized on the internal or external side of the membrane. The hydrogenase has a M_r of 58,000 and appears to consist of a single polypeptide; however, preparations were contaminated with another protein (105). The hydrogenase also contained six nonheme irons and six labile sulfides per 58,000 daltons. The EPR spectrum of the oxidized hydrogenase exhibited a signal at g = 2.02 below 30°K and rhombic signals

with g-values at 2.32, 2.23, and 2.01 that could be observed up to 120°K. The latter were attributed to nickel, but the nickel content of the protein was not reported. No EPR signals were observed when the enzyme was reduced with dithionite. The presence of a three-iron center has been confirmed by resonance Raman spectroscopy (86).

Cytochrome c_3 (M_r = 13,000) from the bacterium was utilized as electron donor in the evolution assay, and the hydrogenase appeared to be reversibly inactivated by oxygen. As with other hydrogenases from *Desulfovibrio*, inhibition by CO was completely reversible, and sensitivity to the inhibitor was comparable to that observed with the *D. gigas* hydrogenase. Except for the low nonheme iron value and molecular weight, which may be due to an impure protein, the membrane-bound hydrogenase of *D. desulfuricans* appears to be similar to the (NiFe) hydrogenase from *D. gigas*.

Conclusion

Two types of hydrogenase, as shown in Table II, have been isolated from various strains and species of *Desulfovibrio:* one, the (Fe) hydrogenase, has high specific activity in the evolution assay, contains only nonheme iron, consists of a single polypeptide chain, and exhibits high sensitivity to CO inhibition; the other, the (NiFe) hydrogenase, has low activity in the evolution assay, contains redox active nickel and nonheme iron, consists of two subunits, has a three-iron cluster in the oxidized state, and exhibits a lower sensitivity to CO inhibition (Table II). Both hydrogenases appear to be periplasmic enzymes and to utilize cytochrome c_3 (M_r = 13,000) as electron carrier, but they have not both been isolated from the same strain or species. The (Fe) hydrogenase is similar to the hydrogenases of *M. elsdenii* (186) and *Clostridium pasteurianum* (44), a notable difference being oxygen sensitivity and, in the case of *C. pasteurianum,* a cytoplasmic localization. The (NiFe) hydrogenase shows similarities with the hydrogenases of *Methanobacterium thermoautotrophicum* (9), *Chromatium vinosum* (10), and *W. succinogenes* (182).

As yet, it has not been possible to isolate a hydrogenase from *Desulfovibrio,* which is definitively localized in the cytoplasm or on the internal surface of the cytoplasmic membrane as required by the idea of hydrogen cycling. According to our present concepts of protein transport (164), the cytoplasmic hydrogenase should have different physical properties from the periplasmic hydrogenase; multiple forms of hydrogenase have been reported in *Desulfovibrio* (103, 125, 209). The biological significance of the two different periplasmic hydrogenases in the same genus is obscure. The two hydrogenases have about the same K_m values for hydrogen, and bacteria that have one or the other hydrogenase compete effectively for growth by interspecies hydrogen transfer (158). In contrast, the hydrogenases of the methanogens have a higher K_m for hydrogen than the hydrogenases of the SRB (98). It does not appear logical that the two

Table 2 Properties of hydrogenases from *Desulfovibrio*

Property	*D. vulgaris* (Hildenborough)	*D. gigas*	(ATCC 27774)	*D. desulfuricans* (NRC 49001)	(Norway 4)
Localization	periplasm	periplasm	NR[1]	periplasm	membrane
Molecular weight	49,000	89,500	77,600	52,000	58,000
Subunits	1	2	2	1	1
Nickel	0	1	1	NR	(by EPR)
Nonheme iron	12	12	12	12	6
$[Fe_3S_3]$	0	1	1	NR	(g = 2.02)
$[Fe_4S_4]$	3	2	2	NR	NR
Specific Activity					
Evolution	4600	440	152	3800	65
Consumption	50,000	NR	NR	1050	200
References	(80, 127)	(72, 41, 118)	(103)	(58)	(86, 105)

[1]Not reported
[2]μmol H_2/min/mg protein

hydrogenases are the result of specific mutation, but the biosynthesis of one or the other hydrogenase might be the result of the availability of metals, which is difficult to control during the growth of *Desulfovibrio* because of the production of sulfide.

ELECTRON-TRANSFER PROTEINS

Cytochrome c_3 *(M_r = 13,000)*

D. VULGARIS (HILDENBOROUGH) The cytochrome c_3 from *D. vulgaris* has a M_r of 13,000 and contains four nonequivalent hemes per molecule. The cytochrome is autooxidizable and in the oxidized state is resistant to reaction with exogenous ligands. In the reduced form it is reactive towards exogenous ligands, and reaction with imidazole, cyanide, and nitric oxide induces EPR signals characteristic of the heme ligand (46). Histidine is coordinated to each heme iron in the fifth and sixth ligand position. The four heme are in nonequivalent sites, as indicated by EPR spectroscopy, and exhibit four different oxidation-reduction midpoint potentials (−284 mV, −310 mV, −319 mV, and −324 mV) with n values near 1 (47). Similar data has been reported for cytochrome c_3 from one of the Japanese strains of *D. vulgaris* and gave midpoint potential values of −266 mV, −278 mV, −298 mV, and −339 mV (211), but there are microscopic interactions between each of the hemes (135, 188).

The amino acid sequence of the cytochrome has been determined (12, 180); but the three-dimensional structure of the cytochrome from the Hildenborough strain has not been reported. X-ray crystallographic studies have been completed on a cytochrome c_3 isolated from one of the Miyasaki strains of *D. vulgaris* at 2.5-Å (76) and 1.8-Å (77) resolution. Overall dimensions of the molecule are approximately 33 × 39 × 34 Å, and the heme-heme distances vary from 11.3 to 18.1 Å. The hemes are found in a compact cluster and differ in their exposure to the external environment. It has not yet been possible to assign E_o' values to specific hemes revealed by the three-dimensional structure. Other physical properties of the cytochromes c_3 have recently been reviewed (211) and will not be discussed in detail.

Cytochrome c_3 is the naturally occurring electron carrier for hydrogenase (206) and is found among the periplasmic proteins in a ratio of 4.5 cytochromes per hydrogenase molecule (127); however, this ratio may be expected to vary due to variability in the amounts of hydrogenase. The cytochrome c_3 has also been reported to be periplasmic in *D. vulgaris* (Marburg) growing on hydrogen and sulfate (18). Although the absence of cytochrome c_3 from the cytoplasmic proteins has not been rigorously demonstrated, it appears highly unlikely that the cytochrome can be both a cytoplasmic and periplasmic protein and therefore should not be considered as general electron carrier in the metabolic

reactions of *Desulfovibrio*. The cytochrome has been reported to stimulate the phosphoroclastic reaction (5) and hydrogen utilization with various substrates (83, 143), but we suggest that these stimulations are largely fortuitous, perhaps due to increased electron flow afforded by the presence of the periplasmic hydrogenase system among the cytoplasmic proteins. Exceptions to this proposal appear to be the reduction of elemental sulfur and hydroxylamine. Although *D. vulgaris* is unable to grow with elemental sulfur as a terminal electron acceptor, the reduction of colloidal sulfur was reported by cytochrome c_3 in early studies on the function of the cytochrome (83).

Recently, it has been confirmed that the cytochrome c_3 from *D. vulgaris* can reduce elemental sulfur but that the product, sulfide, strongly inhibits the reaction (54). This interesting observation probably accounts for the inability of the bacterium to grow with elemental sulfur as terminal electron acceptor and may also account for the inhibition of growth with sulfate observed with high sulfide concentrations. In the presence of dithionite, hydroxylamine is reduced to ammonia by the cytochrome c_3 of *D. vulgaris;* however, the reduction is not a reversible reaction because the four hemes of the reoxidized cytochrome could not be completely reduced, indicating that the cytochrome was damaged by intermediates in the reduction (46).

D. GIGAS Cytochrome c_3 isolated from *D. gigas* has a M_r of 13,000 and contains four heme but differs from that of *D. vulgaris* in having an alkaline (pH 10.5) isoelectric point rather than an acidic one (pH 5.2) (110). The sequence of the cytochrome has been determined, and it has about 50% homology with the cytochrome from *D. vulgaris* (14). This cytochrome is also autooxidizable and reactive towards exogenous ligands only in the reduced state (46). Cytochrome c_3 of *D. gigas* shows by EPR the presence of only two low-spin heme resonances with g_2 values at 2.959 and 2.853. No significant changes in line shape or signal width was observed with either heme signal during EPR potentiometric titrations; however, the titration data showed two distinct slopes for each heme signal and the E_o' for the four hemes were estimated (−235 mV, −315 mV, −235 mV, and −306 mV) (205).

The redox cycling of the cytochrome has been studied by monitoring changes in the heme methyl resonances by NMR spectroscopy (162). By this technique, heme-heme interaction of each of the four heme were followed as a function of pH and the redox state of the protein. Their data indicate a more dynamic model of cytochrome c_3 with interactions between all four hemes from −50 to +70 mV and a pH dependence of some of the midpoint and interacting potentials. From these studies, cytochrome c_3 appears to be a highly versatile molecule capable of donating or accepting 1–4 electrons and interacting with a variety of redox couples by modulation of its midpoint redox potentials. Because of effects of pH, it also has the potential for being involved in the

generation of proton gradients, as has been postulated for cytochrome oxidase (199, 172).

The cytochrome c_3 has also been shown to be the electron carrier for hydrogenase and is found among the periplasmic proteins; however, quantitative studies on the localization of cytochrome c_3 in *D. gigas* have not been reported. In crude extracts, cytochrome c_3 does not stimulate the reduction of thiosulfate with hydrogen and only slightly stimulates the reduction of sulfite under the same conditions (70). As other electron transfer proteins stimulated one or both of these reactions, either the cytochrome was not limiting in these extracts or not involved in the electron-transfer sequence.

D. gigas is capable of growth, utilizing elemental sulfur as its terminal electron acceptor (29). Hydrogenase and cytochrome c_3 from *D. gigas* catalyze the biphasic reduction of elemental sulfur to sulfide without inactivation of the cytochrome, as occurs with the cytochrome c_3 from *D. vulgaris* (54). Membrane vesicles (inverted) prepared from *D. gigas* were shown to catalyze electron transfer–coupled phosphorylation in the presence of elemental sulfur and hydrogen (56). Because it would not be anticipated that the elemental sulfur would be transported across the membrane, the observation suggests that cytochrome c_3 or a species of cytochromes c_3 is transmembraneous. In this regard it may be important to note that in contrast to other cytochromes, anhydrous films of cytochrome c_3 conduct electrons quite freely (170), and interactions between periplasmic and membrane-bound cytochromes might be expected (94).

D. DESULFURICANS (ATCC 27774) Cytochrome c_3 from this strain of *D. desulfuricans* has not been localized or extensively studied. In our laboratory, the levels of cytochrome c_3 in sulfate- and nitrate-grown cells were compared, and the amounts of cytochrome c_3 (M_r = 13,000) were essentially the same under both conditions of growth. These observations indicate that the biosynthesis of cytochrome c_3 is constitutive or that cytochrome is required for the reduction of nitrate or nitrite.

D. DESULFURICANS (NORWAY 4) Cytochrome c_3 from the Norway strain of *D. desulfuricans* has a M_r of 16,000, and its sequence and X-ray crystallographic properties have been reported (66). The amino acid sequence of the cytochrome has only about 28% homology with that of *D. vulgaris* (12) and contains three cys-x-y-cys sequences and one cys-A-B-C-D-cys-sequence rather than two of each, as are found in the cytochromes of *D. vulgaris* and *D. gigas*. A similar arrangement of the cysteine residues has also been found in the cytochromes c_3 from *D. desulfuricans* (NCIB 3880) and *D. salexigens* (13, 15). The EPR spectra of the four hemes have been resolved by their different *g* values and redox potentials (42). As suggested by earlier studies (205), two of the hemes appear to be high potential, both −125 mV, and two of lower potential, −305 mV and −325 mV. In terms of the tertiary structure, the grouping of the midpoint potentials of the hemes probably reflects their topology. Two of the hemes lie in a groove and are exposed to the external medium. These are probably the low-potential hemes. The other two hemes, probably high potential, are localized in less-exposed areas of the molecule (66, 77).

CONCLUSION The low-potential tetraheme cytochromes c_3 (M_r = 13,000) are found only in SRB belonging to the genus *Desulfovibrio* and occur as constitutive periplasmic proteins. Their prime function is as an electron carrier for the periplasmic (NiFe) and (Fe) hydrogenases. Hydrogenases from other anaerobic bacteria are specific for ferredoxin (3) and do not oxidize or reduce cytochrome c_3. A secondary function appears to be the reduction of elemental sulfur to sulfide. Each heme is bound to the protein by two thioether bonds involving cysteine residues, and the fifth and sixth ligands of each heme iron are histidine residues. The four hemes are localized in nonequivalent protein environments as indicated by EPR and NMR spectroscopy, and each heme can exhibit macroscopic individual redox midpoint potentials. It appears that two of the hemes are exposed to the external medium and tend to be low potential, whereas the other two hemes are less exposed and tend to be of higher potential. Although the cytochromes isolated from different species exhibit only about 25% homology, the arrangement of the heme cluster is conserved in the two cytochromes for which the three-dimensional structures are available.

Ferredoxins

D. VULGARIS *D. vulgaris* strains Hildenborough (171) and Miyazaki (81) synthesize only small amounts of ferredoxin even when grown in media containing excess iron; however, these bacteria do produce large amounts of flavodoxin. Knight & Hardy (95) first noted that ferredoxin is not produced by *C. pasteurianum* in iron-depleted media but is replaced by flavodoxin, which can replace ferredoxin in most of its redox reactions. The iron nutrition of the SRB is difficult to analyze because the reduction of sulfate to sulfide results in the formation of insoluble ferrous sulfide. It is thus not evident whether the low levels of ferredoxin reflect iron nutrition or an innate property of *D. vulgaris*. Regardless of the mechanism, one can conclude that in cells of *D. vulgaris* grown under normal laboratory conditions, little ferredoxin is available for cytoplasmic electron-transfer reactions and is replaced by flavodoxin.

D. GIGAS *D. gigas* biosynthesizes both flavodoxin and ferredoxins under normal conditions of growth, and its ferredoxin has been extensively studied. Ferredoxin was initially shown to have a M_r of 6000 and to contain only six

cysteine residues and one (Fe_4S_4) cluster per molecule (104, 111). Its amino acid sequence has been determined and has confirmed these initial observations (179, 33). Subsequent investigations revealed that the ferredoxin could be separated by chromatographic procedures into two components (ferredoxin I and ferredoxin II) containing the same monomeric unit described above (37). Ferredoxin I was shown to be a trimer and ferredoxin II a tetramer. Small amounts of a third oligmeric form of the ferredoxin (ferredoxin I') were also isolated, but this appears to be a mixture of ferredoxin I and II.

Ferredoxin I is diamagnetic in its oxidized state but on reduction exhibits a $g = 1.94$ EPR signal typical of ferredoxin-type (Fe_4S_4) centers. Ferredoxin II is paramagnetic in its oxidized state and exhibits a strong $g = 2.02$ EPR signal, but on reduction the ferredoxin is diamagnetic (40). Ferredoxin I clearly contains a ferredoxin-type (Fe_4S_4) cluster and has a low redox potential ($E^{o'} = -440$ mV). The ferredoxin II was demonstrated to contain three iron atoms per monomeric subunit (79) and to have a higher redox potential (−130 mV). By Mössbauer and EPR studies, ferredoxin II was shown to contain a three-iron cluster similar to that found in aconitase and ferredoxin from *Azotobacter vinelandii* (90); however, it should be noted that the ferredoxin from *A. vinelandii* has a much lower redox potential (−420 mV) than ferredoxin II.

Considerable controversy has arisen over the exact nature of the three-iron center (25), and it now appears that the three-iron center contains four labile sulfides (85); however, problems still exist with the bond distances and core geometry. The two ferredoxins also differ in their biological reactivities (133). Ferredoxin I containing the four-iron center is active in catalyzing the production of hydrogen from pyruvate in the phosphoroclastic reaction but is not effective in promoting the oxidation of hydrogen with sulfite in ferredoxin-depleted extracts. Ferredoxin II is active in stimulating the oxidation of hydrogen with sulfite in these same extracts but is not initially active in stimulating the production of hydrogen from pyruvate; however, after a 30 min lag in the presence of pyruvate, ferredoxin II begins to stimulate hydrogen production from pyruvate. At the same time, a loss of the $g = 2.02$ EPR signal is observed, due to the three-iron center and an appearance of $g = 1.94$ signal indicative of the (Fe_4S_4) ferredoxin-type cluster (117).

These results show that extracts of *D. gigas* are converting three-iron centers to four-iron centers in the presence of pyruvate. Using a combination of EPR and Mössbauer spectroscopy, chemical interconversions of the four- and three-iron centers of ferredoxin I and ferredoxin II have been observed (91). When the apoprotein of the ferredoxin is incubated with excess sulfide and ferrous ions, the four-iron center is reconstituted, but when the apoprotein is incubated with stoichiometric amounts of sulfide and ferrous ion, ferredoxin II containing the three-iron center is formed. Incubation of ferredoxin I with ferricyanide also results in the formation of ferredoxin II.

The unexpected ease of these interconversions poses the question as to whether three-iron centers have physiological significance or simply result from the random oxidation of four-iron centers. The reductive activation of beef heart aconitase (92) by the conversion of a three-iron center to a four-iron center does not necessarily indicate a physiological function for three-iron centers, because they could be an artifact of isolation procedures. The change in specificity that occurs during the interconversions of ferredoxins I and II from *D. gigas* does support the idea that three-iron centers are of physiological significance. In terms of hydrogen cycling, there is a necessity for the separation of electron transfer leading to hydrogen production from that leading to hydrogen utilization, and the bioconversion of nonheme iron centers may represent a mechanism for regulation of the levels of specific electron carriers.

D. DESULFURICANS (NORWAY 4) Two types of ferredoxin have been isolated from the Norway strain of *D. desulfuricans* that, although named ferredoxin I and ferredoxin II, differ significantly from the ferredoxins of *D. gigas* (38). Both ferredoxins have a M_r of 12,000 and consist of two identical subunits of 6,000 daltons; however, the subunits are different, as indicated by amino acid sequence analysis of their N-terminal peptides. Based on these limited sequence analysis, the ferredoxin I appears to be homologous to the monomer of *D. gigas* ferredoxin, whereas the ferredoxin II appears to be more homologous to the ferredoxin of *C. pasteurianum* (61). Ferredoxin I contains six cysteine residues and one (Fe_4S_4) cluster. As isolated, the ferredoxin exhibits no significant EPR signal but on reduction with dithionite yields a typical $g = 1.94$ signal with a midpoint potential of −374 mV at pH 8.0 (63). Ferredoxin II, the more abundant protein, is labile to oxygen; its iron and sulfide contents have not been reported. Although it contains only six cysteine residues, its extinction coefficient and EPR spectra are consistent with the presence of two (Fe_4S_4) clusters.

The EPR spectrum of oxidized ferredoxin again did not show significant signals, but on reduction with limiting dithionite an atypical $g = 1.94$ signal was seen that in the presence of excess dithionite became a typical $g = 1.94$ signal with a midpoint potential of −500 mV. The intermediate form had a midpoint potential of −440 mV and was assigned to two interacting (Fe_4S_4) clusters. These ferredoxins are both low potential and are active in stimulating hydrogen production in the phosphoclastic reaction and the oxidation of hydrogen with sulfite in ferredoxin-depleted extracts of Norway 4. It appears that *D. desulfuricans* (Norway 4) contains two low-potential ferredoxins, one of the four-iron type and the other of the eight-iron type, that are equally active in hydrogen uptake and evolution.

D. AFRICANUS Three different types of ferredoxins have been isolated from extracts of *D. africanus:* ferredoxin I, the major ferredoxin; ferredoxin II (73); and ferredoxin III, isolated from cells grown on media containing high iron

(74). Ferredoxin I, II, and III were shown to have molecular weights of 11,000, 9,600, and 15,000 and to be dimers composed of identical subunits with minimal molecular weights of 6,838, 5,828, and 7,500. Ferredoxins I and II are similar in that they contain four nonheme irons and stimulated hydrogen production in the phosphoroclastic reaction, but have different amino acid compositions. Analysis of their N-termnal amino acid sequenced indicated that the two ferredoxins are different from but homologus to the ferredoxin of *D. gigas*. The complete amino acid sequence of ferredoxin has been reported (36a). The ferredoxin III contains six cysteine residues but has 12–13 g atoms of nonheme iron and sulfide per monomeric unit.

This result is curious but in agreement with the extinction coefficient of the ferredoxin. Analysis of the amino acid sequence of the N-terminal amino acid residues showed that the ferredoxin was different from ferredoxin I and II, and suggests that it is homologous to the ferredoxin of *C. pasteurianum*. Ferredoxin III exhibited low activity in both the hydrogen uptake assay with sulfite and the phosphoroclastic reaction.

CONCLUSION There does not appear to be a consistent pattern in the physical properties of the ferredoxins found in the various species of *Desulfovibrio*. This variation could be due to nutritional effects on the biosynthesis of the ferredoxins or technical problems in the isolation of the ferredoxins; however, variations in the levels of its counterpart, flavodoxin, and the unique oligomeric ferredoxin system in *D. gigas* suggest that these variations are real. The available data indicate that the ferredoxins are all cytoplasmic proteins and, except for the ferredoxins of *D. gigas*, are all active in stimulating hydrogen evolution from pyruvate and hydrogen utilization with sulfite. In order to complement data on the ferredoxin system of *D. gigas*, more information is required on structures and specificities of the new ferredoxins before a logical picture of their roles in the bioenergetics of these species can be developed.

Flavodoxins

Flavodoxin is not found in all of the species of *Desulfovibrio* and, where it is absent, seems to be replaced by an eight-iron clostridial-type ferredoxin (Table 1). The flavoprotein has been shown to have the ability to replace ferredoxin in both H_2-producing and H_2-utilizing reactions (70, 81, 84, 171). Similarity in biological specificity is also indicated by the fact that flavodoxin replaces ferredoxin in *C. pasteurianum* grown on iron-depleted media (95). Flavodoxin is not found among the periplasmic proteins of *D. vulgaris* (127) and must be regarded as a soluble cytoplasmic protein. Both the amino acid sequence (52) and tertiary structure (193) of the flavodoxin of *D. vulgaris* (Hildenborough) have been determined and compared with the x-ray crystallographic structure of the flavodoxin from *Clostridium* MP (126).

It is interesting that many of the differences between the two flavoproteins are found around the isoalloxazine ring of the FMN. The flavodoxins from three species of *Desulfovibrio—D. vulgaris, D. gigas,* and *D. salexigens*—have been examined by NMR spectroscopy and shown to exhibit significant differences (57). Both methyl resonances of FMN can be observed with the flavodoxin from *D. vulgaris* in the oxidized and reduced states, indicating the dimethylbenzene end of the FMN is exposed to solvent; however, with the reduced flavodoxin from *D. gigas,* only one methyl signal is seen, suggesting that the flavin is more deeply buried in the protein. In the case of the flavodoxin from *D. salexigens,* no methyl resonances are observed, indicating that the flavin is even more deeply buried in the molcular. No differences in the biological specificities of these molecules have been reported.

There exists an interesting redox analogy between flavodoxin and the oligomeric ferredoxin system of *D. gigas*. Flavodoxin exhibits two stable redox states; the blue semiquinone-hydroquinone form ($E_o' = -440$ mV) and the colorless semiquinone-quinone form ($E_o' = -150$ mV). The midpoint potentials of flavodoxin are similar to those of ferredoxin I ($E_o' = -440$ mV) and ferredoxin II ($E_o' = -130$ mV); however, this comparison can not be made with the ferredoxins found in other species of *Desulfovibrio*. The two redox states suggested the possibility that flavodoxin can also catalyze specific electron transfer as a function of its redox state. In this regard it has been reported (152) that flavodoxin can substitute for ferredoxin with the NADH-ferredoxin oxidoreductase of *C. tyrobutyricum,* but the flavodoxin is only reduced to the blue hydroquinone form in the absence of acetyl CoA.

Alternatively, specificity may result in part from the cellular localization of enzymes. Flavodoxin and ferredoxin both stimulate the oxidation of hydrogen with sulfite in preparations of *D. gigas* containing inverted membrane vesicles and soluble proteins (both periplasmic and cytoplasmic), but only ferredoxin supports electron-transfer phosphorylation. These observations suggest the reduction of both ferredoxin and flavodoxin by the periplasmic hydrogenase mixed with the cytoplasmic proteins. Specificity for phosphorylation could be introduced by the reduction of only ferredoxin II by the periplasmic hydrogenase system contained within the membrane vesicles (21).

In conclusion, flavodoxin has not been found to occur in all species of *Desulfovibrio* and generally appears capable of replacing ferredoxin in electron-transfer reactions. Although there is considerable molecular diversity among these flavodoxins, it is not reflected as specificity in the reactions where flavodoxin is active.

Rubredoxins

Rubredoxins have low molecular weight, around 6,000, and contain one iron ligated to the sulfhydryl groups of four cysteine residues. They appear to be

cytoplasmic proteins and, thus far, have been found in all species of *Desulfovibrio*. The physiological function of rubredoxin in the SRB remains unknown, although a NADH:rubredoxin oxidoreductase has been reported in *D. gigas*. The enzyme has a high degree of specificity for the rubredoxin from *D. gigas* ($K_m = 6.2 \times 10^{-6}$M) compared to the rubredoxins from *D. vulgaris* ($K_m = 5.3 \times 10^{-5}$M) and *C. pasteurianum* ($K_m = 1.0 \times 10^{-4}$M) (116). The amino acid sequences of the rubredoxins from *D. vulgaris* and *D. gigas* (36) have been established, and the tertiary structures of the protein from *D. vulgaris* and *D. desulfuricans* (ATCC 2774) have been determined (4, 162a).

For members of the same genus, there are many more differences in the sequences than might be expected: of 52 residues, only 37 are identical. The same amount of sequence differences exist between the rubredoxins of *C. pasteurianum* and *D. vulgaris*. With regard to the tertiary structure, no major differences in conformation or internal hydrogen bonding between the rubredoxins from *D. vulgaris* and *C. pasteurianum* occur, and their structures differ largely in groups on the surface of the molecules.

Cytochrome c_{553}

A low-molecular-weight (9000 daltons) monoheme cytochrome has been isolated from *D. vulgaris* (Hildenborough) (112), *D. vulgaris* (Miyazaki) (207), and *D. desulfuricans* (Norway 4) (55) but is absent in *D. gigas* (34) and *D. desulfuricans* (ATCC 27774) (122). The cytochrome isolated from the Norway strain has a double α absorption and has been termed cytochrome $c_{553(550)}$; these monoheme cytochromes are autooxidizable and have redox potentials around +10 mV. The presence of an absorption band at 695 nm suggests methionine rather than histidine as the sixth coordination ligand of the heme iron. The primary structures of cytochrome c_{553} from *D. vulgaris* (Hildenborough) and (Miyazaki) have been reported (35, 124a), and the cytochrome can be distantly related to the mitochondrial cytochrome *c* family (11).

Cytochrome c_{553} is reduced by partially purified preparations of formate dehydrogenase (207) and D-lactate dehydrogenase (140) from *D. vulgaris* (Miyazaki), and it was proposed as the natural electron acceptor for these dehydrogenases. Formate dehydrogenase is a periplasmic protein, (138), as is cytochrome c_{553}, and could function as the immediate electron acceptor for formate dehydrogenase; however, the cytochrome has not been detected in all species of *Desulfovibrio*, and the low-potential cytochrome c_3 ($M_r = 13,000$) is required for the formate-hydrogenlyase reaction of *D. vulgaris* (82). The high redox potential of the cytochrome c_{553} probably prohibits it from being involved in this reaction. In the case of *D. gigas*, cytochrome c_3 ($M_r = 13,000$) is reduced by formate dehydrogenase (148), and it may function as an alternate electron carrier. In contrast to formate dehydrogenase, D-lactate dehydrogenase is a cytoplasmic dehydrogenase and the cytochrome c_{553} is physically separated from the dehydrogenase. Thus, a definitive role for the monoheme cytochrome in the SRB has not been established.

Cytochrome c_3 *(M_r = 26,000)*

Cytochrome c_3 ($M_r = 26,000$) was first isolated from *D. gigas* (34) and termed cytochrome cc_3. It has been found in all species of *Desulfovibrio* investigated. It was shown to contain eight hemes per 26,000 daltons, a low redox potential, and to have an amino acid composition that distinguishes the cytochrome from cytochrome c_3 ($M_r = 13,000$). The latter result also seemed to eliminate the possibility that it was a dimer of cytochrome c_3 ($M_r = 13,000$). Preliminary optical and EPR studies on the octaheme cytochrome indicate that the fifth and sixth ligand positions of the hemes are occupied by histidine and that some hemes are in nonequivalent environments (113).

Further investigations into the nature of the cytochrome have been limited, due to the low solubility of the protein in aqueous media. Cytochrome c_3 ($M_r = 26,000$) was recently isolated and characterized from *D. desulfuricans* (Norway 4) (62). By removing the hemes prior to SDS electrophoresis, it was shown to be a dimer consisting of two identical subunits of 13,500 daltons. The N-terminal sequence of the subunit was determined, and no homology with the N-terminal sequence of cytochrome c_3 ($M_r = 13,000$) was observed. A cytochrome similar to the high-molecular-weight cytochrome c_3 has been isolated from *D. desulfuricans* (ATCC 27774) and shown to have a M_r of 40,800 with 12 hemes per molecule. Superfically, it would appear to be a composed of three subunits of the cytochrome c_3 ($M_r = 13,000$); however, subunits could not be detected even after removal of the heme groups (122).

Cytochrome c_3 ($M_r = 26,000$) of *D. gigas* reacts with hydrogenase in a very similar fashion to the lower-molecular-weight cytochrome c_3. It is active in the evolution of hydrogen from dithionite and has a Km value 1×10^{-6} M, comparable to that of cytochrome c_3 ($M_r = 13,000$) (27). The high-molecular-weight cytochrome can also be reduced by hydrogenase and, in crude extracts of *D. gigas*, stimulates specifically the reduction of thiosulfate. With pure hydrogenase and thiosulfate reductase (67), maximum activity was obtained in the presence of both multiple heme cytochromes (70). Cytochrome c_3 ($M_r = 26,000$) has not been found among the periplasmic protelns but is present in crude extracts in a slightly lower concentration than the smaller cytochrome c_3. On the basis of the information available to date, it appears that cytochrome c_3 ($M_r = 26,000$) is a good candidate for the natural electron carrier for the proposed cytoplasmic hydrogenase, because the reduction of thiosulfate does not appear to be coupled to vectorial electron transfer (96).

Other Redox Proteins

A number of interesting redox proteins has been isolated and studied from various species of *Desulfovibrio* but will not be discussed because the function and distribution in *Desulfovibrio* have not been established. These include a membrane-bound cytochrome *b* (69, 87), a molybdenum-containing iron sulfur protein (M_r = 120,000 daltons) (132), desulforedoxin (131), cobalt porphyrin–containing protein (M_r = 11,000) (68, 134), a "split-Soret" cytochrome containing two subunits and four hemes (122), and a new type of iron protein (165).

Conclusion

From this consideration of electron-transfer proteins, it is possible to make a number of generalizations. The presence of a periplasmic hydrogenase and cytochrome c_3 (M_r = 13,000) appear to be a constant characteristic of *Desulfovibrio*. At the molecular level, there have been extraordinary changes in the primary structure of cytochrome c_3, but the heme arrangement and general properties of the molecule have been conserved. The monoheme cytochrome is lacking in some species and thus does not appear to play an essential role in sulfate reduction.

Only rubredoxin and cytochrome c_3 (M_r = 26,000) are found in significant amounts in all the species of *Desulfovibrio*. Flavodoxin is lacking in several species. The level and number of ferredoxins vary considerably, but each species investigated in detail seems to form at least two molecular species of the nonheme iron protein. Except for the ferredoxins from *D. gigas*, these electron-transfer proteins exhibit little specificity for either hydrogen production or utilization. The information generated on electron transfer in *D. gigas* cannot be directly applied to other species; therefore the cytoplasm pathways for electron transfer should be investigated in different species as separate problems.

BIOENERGETICS

Proton Translocation

The demonstration of the existence of proton translocation requires that proton extrusion across a chemisomotic membrane be in excess of proton changes that can be derived from scalar or substrate-level production of protons. The occurence of proton translocation coupled to the oxidation of hydrogen with nitrite has been extensively studied with intact cells of nitrate-grown *D. desulfuricans* (ATCC 27774) (169). The proton-releasing site of hydrogenase was shown to be on the external aspect of the cytoplasmic membrane as is the proton-consuming site of the nitrite reductase, a c-type hexaheme protein

(121). Thus, the scalar reaction shown in Reaction 1 results in the consumption of two protons on the periplasmic aspect of the cytoplasmic membrane.

$$3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2H_2O \qquad 1.$$

Utilizing starved cells to eliminate endogenous acidification of the medium, an acidification rather than an alkalization of the external medium equivalent to the translocation of four protons per nitrite reduced to ammonia was observed. Correcting this data for proton consumption an $H^+/2e$ value of 2.2 was obtained. Optimal conditions for proton translocation were established by varying the concentration of the permeant anion, KSCN, pH, and the size of the nitrite pulse. Addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone led to immediate collapse of the proton gradient and demonstrated the chemiosmotic nature of the membrane. Proton translocation was also observed with hydrogen and nitrate; however, the rate of proton translocation was slower than that observed with nitrite. In addition, it was not possible to assess the location of the proton-consuming site of the nitrate reductase relative to the cytoplasmic membrane. Nevertheless, assuming an external scalar utilization of two protons per nitrate reduced, a minimum $H^+/2e$ ratio of 1.6 for the eight-electron reduction of nitrate was calculated.

$$NO_3^{-1} + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O \qquad 2.$$

The results establish that proton translocation is coupled to the reduction of nitrate to ammonia, but it is not clear whether proton translocation is coupled to the reduction of nitrate to nitrite. Generation of a proton gradient was also observed with endogenous substrate in freshly harvested cells and with lactate and formate in starved cells after pulses of nitrite, but the mechanisms involved in the generation of the proton gradient could not be evaluated. The proposed relationships for proton translocation coupled to hydrogen oxidation with nitrite are shown in Figure 1.

Sulfate-grown cells of *D. desulfuricans* are also capable of generating a proton gradient during the oxidation of hydrogen with nitrite, producing a $H^+/2e$ ratio of about 2.0. Similar results were obtained with *D. gigas*, which has not been shown to utilize nitrate in place of sulfate as a terminal electron acceptor for growth (24). Cell-free preparations of *D. gigas* can couple the formation of ATP to the oxidation of hydrogen with nitrite or hydroxylamine. With nitrite, P/H_2 ratios were similar to those observed with fumarate, about 0.3–.4, but with hydroxylamine, the ratio was around 0.1. In both systems, phosphorylation was sensitive to uncoupling agents. The fact that sulfate-grown cells of *D. desulfuricans* and *D. gigas* catalyze proton translocation

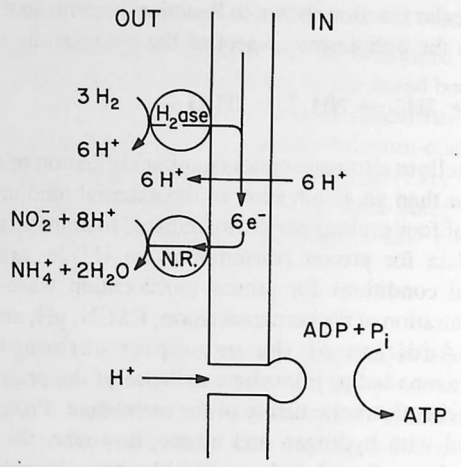


Figure 1 Scheme for proton translation coupled to the oxidation of hydrogen with nitrite.

coupled to hydrogen oxidation with nitrite indicates that the overall reaction is not a specific adaptation to growth with nitrate as terminal electron acceptor. In addition to nitrate reductase, nitrate-grown cells show increased levels of nitrite reductase that appears to be localized on the cytoplasmic aspect of the membrane. This in turn suggests the possibility that cytoplasmically generated nitrite is metabolized in a different manner from externally added nitrite.

These observations show that at least two species of *Desulfovibrio* are able to generate a proton gradient by a mechanism other than vectorial electron transfer and associated scalar protein production. Whether the mechanism involves a typical proton redox pump or involves the mechanism of nitrite reductase itself has not been established. In support of the latter idea, the hydrogen/nitrite couple exhibits unique kinetic properties compared to other redox couples, and in vitro there is a close physical association between hydrogenase and the nitrite reductase (168). Recently, it has been reported (172) that cytochrome c_3 (M_r = 13,000) can be inserted into artificial membranes and will catalyze electron transfer between hydrogen (outside) and ferricyanide (inside). The system also catalyzes the transfer of protons from the outside to the inside, but the transfer appears to be in the opposite direction to that which occurs physiologically.

The formation of a proton gradient coupled to the oxidation of hydrogen with sulfite, but not with sulfate, trithionate, thiosulfate, or NaCl (96, 119) has been observed. With starved cells of *D. vulgaris* and *D. desulfuricans* (27774), H^+/2e ratios of around 2.0 were obtained that could be explained by vectorial electron transfer between the electron donor outside (Reaction 3) and the electron acceptor inside (Reaction 4).

$$3H_2 \rightleftharpoons 6H^+ + 6e \quad 3.$$

$$SO_3^{-2} + 6H^+ + 6e \rightarrow S^{-2} + 3H_2O \quad 4.$$

The generation of the proton gradient is sensitive to uncoupling agents, and the number of protons produced assuming $2H^+$/ATP agrees with the predicted number of ATP's formed from growth-yield studies and the available energy from the SO_3^{2-}/H_2 redox couple (42 Kcal/mol). Any consideration of the number of protons translocated must take into account the protons generated in the scalar reactions, and these appear to be about the maximum that could be generated by the redox couple. Higher H^+/2e ratios have been reported with unstarved cells of *D. vulgaris* (96) but this result may be due to partial coupling of proton translocation to the utilization of endogenous substrates. This is suggested by the observation that initially, proton translocation with sulfite was the same in the presence of either hydrogen or nitrogen.

The failure to observe proton translocation coupled to the oxidation of hydrogen with sulfate may be due to a number of factors. First, growth-yield data have been interpreted to indicate that ATP generation is not coupled to the reduction of sulfate to sulfite (17). Second, the reduction of sulfate to sulfite requires ATP (142), and this requirement may complicate interpretation of the proton translocation data. Third, sulfate does not freely cross the cytoplasmic membrane, and its transport may require energy (189). This may result in time lags in the reduction of sulfate with hydrogen. The failure to observe proton translocation coupled to the reduction of thiosulfate and trithionate cannot be explained by the considerations that apply to sulfate. The data on proton translocation suggest that thiosulfate and trithionate may not usually be free intermediates in the reduction of sulfite to sulfide and are reduced in the cytoplasm. Together, the available information indicates that vectorial electron transfer is entirely responsible for the generation of the proton gradient generated during the reduction of sulfite with hydrogen.

Hydrogen Cycling

Laboratory and field studies (1, 2, 20, 30, 45, 123, 128, 141, 200, 202, 203) have shown that the transfer of molecular hydrogen is an integral and vital process in the anaerobic mineralization of organic matter. These hydrogen transfers are generally regarded as interspecies interactions between fermentative hydrogen–evolving organisms and respiratory hydrogen–consuming organisms such as the methanogens in low-sulfate environments and the SRB in high-sulfate environments. Interspecies hydrogen transfer constitutes an electron and proton link between fermentations of acids, alcohols, and sugars with the hydrogen-dependent anaerobic respirations to CO_2 sulfate, nitrate, and

organic acceptors. The presence of hydrogen provides additional energy to respiring bacteria through oxidation, whereas hydrogen removal enhances carbon flow through energy-yielding substrate-level reactions. Evidence suggests that certain *Desulfovibrio* that are known to be able to produce or consume hydrogen can carry out an intracellular transfer of molecular hydrogen between cytoplasmic fermentation and a membrane-bound respiratory chain (139). This recycling of molecular hydrogen within the same organism bestows an energetic and adaptive advantage on the organism.

Theoretically, hydrogen cycling can be effected by any set of hydrogen-evolving and hydrogen-consuming reactions within a single organism. The hydrogenase activities need not be enzymologically distinct or simultaneous but must catalyze operationally reverse reactions. Directionality might be achieved by enzymatic specificity or by coupling identical reversible enzymes to electron donors or acceptors more negative or more positive in redox potential than that of hydrogen. This latter mechanism would only be feasible with compartmentalized reactions. As with interspecies hydrogen transfers, cycling of hydrogen from relatively high-potential electron donors such as lactate or ethanol is feasible if the hydrogen-utilizing reactions are kinetically competent to maintain very low partial pressures of hydrogen. The interposition of a proton-impermeable, hydrogen-permeable membrane between the two hydrogenase activities allows the formation of a proton gradient, provided that there is not consumption or production of protons on the same side of the membrane and that there is no direct electron transfers bypassing the hydrogenase systems. A scheme for hydrogen cycling is depicted in Figure 2.

Lactate enters the cell and is oxidized to pyruvate by lactate dehydrogenases located on the internal aspect of the cytoplasmic membrane or cytoplasm (140, 167, 169). Electrons from the oxidation are utilized to reduce protons to hydrogen. This is a thermodynamically unfavorable reaction and probably occurs to a significant extent only when the partial pressure of hydrogen is maintained at a low level, for example, by interspecies H_2 transfer. Pyruvate is then converted to carbon dioxide, acetyl phosphate, and lower potential electrons that are also used to reduce protons to molecular hydrogen. This segment of the scheme requires an internal hydrogenase (139) and necessitates that electron transfer leading to the production of hydrogen and sulfate be entirely separate from electron transfer leading to the reductases involved in respiratory sulfate reduction. The cytoplasmically produced hydrogen diffuses rapidly across the cytoplasmic membrane and in the absence of an electron acceptor such as sulfate the hydrogen can be utilized by other microorganisms. In the presence of a electron acceptor such as sulfate, the hydrogen is not lost but is reoxidized to protons and electrons by the periplasmic hydrogenase and its cofactor, cytochrome c_3 (M_r = 13,000) (177). The electrons are transferred across the cytoplasmic membrane leaving protons at the external surface of the

BIOENERGETICS OF HYDROGEN METABOLISM IN DESULFOVIBRIO

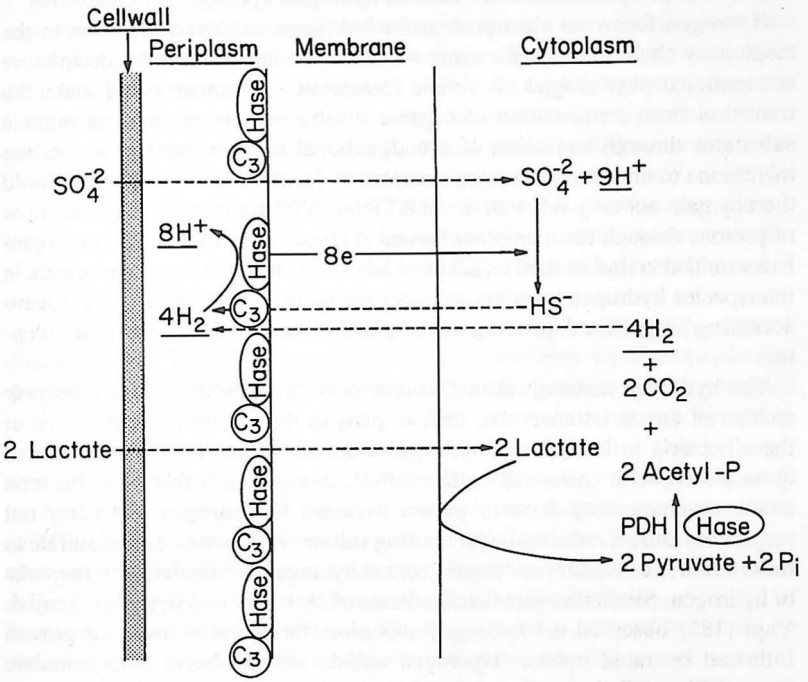


Figure 2 Hydrogen cycling in *Desulfovibrio gigas* (149).

membrane. This establishes the proton gradient and the energy that can be utilized for transport and the synthesis of ATP (60).

In the cytoplasm, the electrons are utilized for the reduction of adenylyl sulfate to sulfide by APS reductase and bisulfite reductase (desulfoviridin). The net effect of hydrogen cycling is the transfer of eight protons from the cytoplasm to the external aspect of the cytoplasmic membrane without direct coupling of proton translocation with electron transfer. In the case of *D. gigas* the scheme is consistent with the available data on the localization of enzymes and electron-transfer proteins (Figure 3), chemiosmotic properties of the cytoplasmic membrane, growth yields, and the general physiology of the bacterium. Direct evidence for hydrogen cycling has been presented by Odom & Peck (139), who showed that spheroplasts of *D. gigas* lacking periplasmic hydrogenase and cytochrome c_3 (M_r = 13,000) are unable to oxidize lactate with sulfate. Addition of purified periplasmic hydrogenase from *D. vulgaris* and

cytochrome c_3 partially restored (40%) this activity. The experiment demonstrated that hydrogen is an essential intermediate in the lactate-sulfate reaction; however, it is not necessary to involve hydrogen in the oxidation of lactate with sulfate except by a mechanism such as hydrogen cycling.

Hydrogen forms an electron-transfer link from oxidative reactions to the respiratory chain in much the same way that pyridine nucleotides do in more conventional physiologies. A simple fermentative organism could make the transition from fermentation of organic substrates to respiration on organic substrates through aquisition of a undirectional electron transfer across the membrane to an internal electron acceptor molecule. Such an organism could thereby gain not only substrate-level ATP but ATP derived from the backflow of protons through the membrane-bound ATPase. A hydrogen cycling organism would also find itself at an adaptive advantage in that it could participate in interspecies hydrogen transfers as either the hydrogen-donating or hydrogen-accepting organism, depending on the presence or absence of electron acceptors.

The hydrogen metabolism of *Desulfovibrio* has been the most intensively studied of any strict anaerobe, due, in part, to the ecological importance of these bacteria in interspecies hydrogen transfer. Vosjan (191) demonstrated quite clearly with chemostat cultures of *D. desulfuricans* that these bacteria could simultaneously ferment excess pyruvate to hydrogen and carry out respiratory sulfate reduction with limiting sulfate. Addition of excess sulfate to these cultures caused either cessation of net hydrogen accumulation or reuptake of hydrogen. Similarly, with batch cultures of *D. vulgaris* (Miyazaki), Tsuji & Yagi (181) observed net hydrogen evolution during early stages of growth followed by rapid uptake. Hydrogen sulfide did not begin to accumulate appreciably until the hydrogen uptake phase commenced. Cultures that had

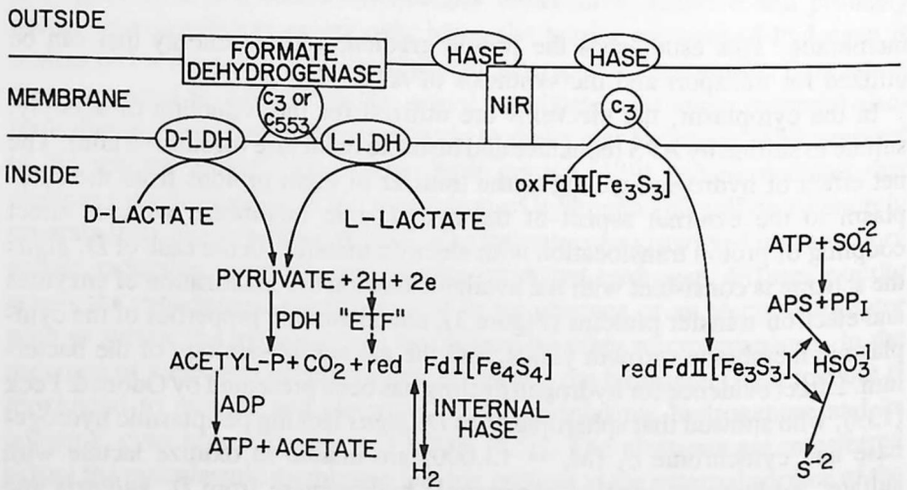


Figure 3 Localization of electron-transfer components and enzymes in *Desulfovibrio gigas*.

been sparged with argon in order to continuously remove hydrogen grew very poorly, which suggested to these workers that hydrogen was important to growth of the cell.

Additionally, two types of membrane-bound hydrogenase, a high-molecular-weight and a low-molecular-weight species were found to correlate with hydrogen evolution and uptake respectively. However, other investigators (93) could discern no "lactate-sparing" effect of hydrogen on *D. vulgaris* (Hildenborough) cultures whereas in contrast, hydrogen was found to completely inhibit lactate oxidation in *D. gigas* cultures (J. M. Odom, unpublished data). Only low levels of hydrogen are usually found in cultures of *Desulfovibrio* (71), but Traore et al (178) detected almost 0.5 mol H_2/mol lactate metabolized in the culture headspace of *D. vulgaris* (Hildenborough). However, in this study as in those by Tsuji & Yagi (181), sulfide did not begin to accumulate until hydrogen evolution had reached its final stages.

Although most *Desulfovibrio* will respire on sulfate with organic electron donors or with hydrogen and also grow fermentatively by interspecies hydrogen transfer, an integrated picture of energy generation has remained unclear. Vectorial electron transfer is sufficient to explain growth by hydrogen uptake, but if a periplasmic reversible hydrogenase is the sole hydrogenase present then severe problems arise when attempting to explain growth by hydrogen evolution and interspecies hydrogen transfer. In such a case: (*a*) The periplasmic hydrogenase must accept electrons via transmembrane electron transfer, because lactate dehydrogenase and pyruvate dehydrogenase are cytoplasmic enzymes; and (*b*) The cytoplasmic oxidation of lactate to acetate and CO_2 results in the liberation of 4–5 H^+, which must be removed from the cytoplasm in order to avoid the development of a chemiosmotic potential opposite to that required for the reversible ATPase and transport.

The export of acetate or the export of protons via a proton-translocating ATPase are mechanisms that might be involved in the export of protons; however, the amount of acetate formed in the reaction is not sufficient to account for the removal of the protons, and the use of ATP for this purpose would deprive the cells of energy. In the latter case the cells would not be expected to grow on lactate by interspecies hydrogen transfer, assuming 2–3 protons/ATP. Alternatively, the oxidation of lactate might be coupled to a redox proton-pump, but this would not be expected on thermodynamic grounds. Given these considerations, it is difficult to envision how the cell could regulate internal pH with periplasmic hydrogen evolution. The best rationale for periplasmic hydrogenase seems to be that of hydrogen oxidation, and it has been postulated that this locale enables the SRB to compete more effectively for transferred hydrogen (26).

The problems inherent in periplasmic hydrogen evolution are obviously circumvented if cytoplasmic oxidations are coupled to an internal hydrogenase,

as is the case in essentially all other phosphoroclastic fermentations studied to date. There are reports of multiple hydrogenases in *Desulfovibrio* sp. (103, 125, 180, 209) but there has yet to be a clear demonstration of a cytoplasmic enzyme. A cytoplasmic enzyme may be difficult to detect when one considers the very high specific activity of the periplasmic enzyme. In addition, hydrogenase activity is, in some cases, partially soluble and partially associated with the membrane, thus making detection of a second—perhaps less active—enzyme difficult. Because of the mechanism of protein export, the cytoplasmic hydrogenase should not be identical to the periplasmic hydrogenase, and it may be rapidly irreversibly inactivated by oxygen, as are the hydrogenases from other anaerobes.

Some indirect evidence other than isolation exists for the presence of a cytoplasmic hydrogenase. When extracts were prepared by lysozyme-EDTA lysis, consistent two- to three-fold increases in crude extract hydrogenase activity over whole-cell rates were observed (139). Detergent lysis with .1% Triton X100 gave identical results with *D. gigas*. In fact, 84% of the whole-cell hydrogenase activity could be removed by buffer washes of intact spheroplasts. Subsequent osmotic lysis of the spheroplast resulted in three-fold stimulation of the residual hydrogenase activity. With *D. desulfuricans* (ATCC 27774), French press lysis resulted in nearly two-fold stimulation of benzyl viologen–linked hydrogenase activity (169). These results are similar to some of the early studies done by Postgate (155), where the detergent CTAB was found to not only stimulate hydrogenase activity but alter electron acceptor specificity as well.

If a cytoplasmic hydrogenase were present, as the latter studies suggest, and involved in growth by interspecies hydrogen transfer and/or hydrogen evolution and recycling, then this activity might be enhanced by growth under low-sulfate conditions. A sulfate-limited chemostat experiment with *D. gigas* clearly showed that, as the dilution rate was lowered and sulfate concentration diminished, the ratio of lysed-cell to whole-cell hydrogenase activity increased dramatically. Very similar results were obtained by selective lysozyme lysis of *D. vulgaris* grown by interspecies hydrogen transfer with *Methanobacterium bryantii*. Here, the increase in activity upon cell lysis was nearly two-fold higher than the increases observed with lactate sulfate–grown *D. vulgaris* (139).

The comparative physiology of hydrogenase in *Desulfovibrio* and *Desulfotomaculum* also yields some interesting insights into hydrogen cycling (120). *Desulfotomaculum* does not have a detectable periplasmic hydrogenase and does not respire with sulfate and hydrogen (119). Instead, sulfate is used as an accessory oxidant in much the same way that *Clostridia* use nitrate as an electron sink (64). However, *Desulfotomaculum* will grow by interspecies hydrogen transfer (194) and extracts exhibit a low level of hydrogenase activity (7) that is bound to the inner aspect of the cytoplasmic membrane (110). Payne & Grant (42) observed that acetylene, an inhibitor of hydrogenase (166), was quite inhibitory to the growth of *D. gigas* and *D. desulfuricans* growing on lactate-sulfate medium. At a range of concentrations from 3% to 20% in the gas phase, the growth of *Desulfovibrio* was either partially or completely inhibited, while growth of *Dt. ruminis* was negligibly effected by acetylene. The result supports the role of hydrogenase in lactate-sulfate respiration by *Desulfovibrio* but not in the lactate-sulfate fermentation of *Desulfotomaculum*.

Conclusion

While the cycling of hydrogen between hydrogenases, as theoretically discussed above, is only currently being resolved in *Desulfovibrio,* another type of hydrogen cycling has been known for many years. This involves the hydrogen transfer relationship between nitrogenase and hydrogenase in nitrogen-fixing bacteria such as the photosynthetic bacteria (89) *Rhizobiaceae* (49) and *Azotobacter chroococcum* (192), and has been termed hydrogen recycling. In these cases, unidirectional uptake hydrogenases are coupled to membrane-bound respiratory chains where proton gradients appear to be formed by proton translocation rather than compartmentation of proton-releasing and proton-consuming reactions.

For example, in *Rhodopsdudomonas capsulata* the hydrogenase appears to be localized on the cytoplasmic aspect of the membrane (89). The hydrogenase of this organism has a very low K_m (0.25μM) for hydrogen (45a). The activity of the enzyme in whole cells is greatly stimulated by the presence of molecular hydrogen during growth under nitrogenase-derepressing conditions. Mutants of this organism that lack uptake hydrogenase evolve more hydrogen than do wild-type cells when grown under conditions where nitrogenase is expressed (113). Dixon (49) has postulated three possibilities for the physiological function of hydrogen recycling in nitrogen-fixing bacteria: (*a*) prevention of nitrogenase inhibition by hydrogen build-up; (*b*) maintenance of low O_2 tension by using hydrogen as a substrate for O_2 reduction; and (*c*) the use of hydrogen as a respiratory substrate for energy production.

Walker & Yates (192) substantiated the second and third postulates in studies on *Azotobacter chroococcum* and additionally showed that hydrogen can be used as an electron donor for acetylene reduction by nitrogenase. However, hydrogen recycling does not appear to be a vital electron-transfer route in nitrogen-fixing bacteria. Hydrogen uptake–deficient mutants of *R. capsulata* display growth properties identical to those of the wild type under nitrogen-fixing conditions (173). Recycling may, however, contribute to the overall energy metabolism of the cell under conditions of electron donor and nitrogen limitation (89).

The strictly anaerobic dissimilatory sulfate-reducing bacteria appear to represent a second group of bacteria capable of cycling hydrogen; however, hydrogen cycling may not be restricted to only the SRB. The acetogenic

bacteria that catalyze the net synthesis of acetate from carbon dioxide using hydrogen (Reaction 5) or organic substrates as electron donors are excellent candidates for the presence of hydrogen cycling.

$$4H_2 + 2CO_2 \rightarrow CH_3\,COOH + 2H_2O \quad 5.$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad 6.$$

Acetobacterium woodii grows autotropically by reducing carbon dioxide to acetate with hydrogen (Reaction 5) or heterotrophically on carbohydrates (Reaction 6) with the production of the extra acetate from carbon dioxide (19).

The data demonstrate that the generation of net ATP as reflected by growth can be derived from the oxidation of hydrogen and reduction of carbon dioxide to acetate. This observation also explains earlier data regarding anomolous growth yields of acetogenic bacteria growing on carbohydrates (16). Because of the similarity of the reduction of carbon dioxide to acetate with hydrogen to the oxidation of hydrogen with sulfate, nitrate, or fumarate, one can speculate that the generation of a proton gradient occurs by means of vectorial electron transfer in these microorganisms. The importance of hydrogen in the heterotrophic growth of *A. woodii* is indicated by the demonstration that two rather than three acetates are produced from fructose when it grows in syntrophic associations with various methanogens (201). A postulated scheme for hydrogen cycling in *A woodii* and possibly other acetogenic bacteria is presented in Figure 4.

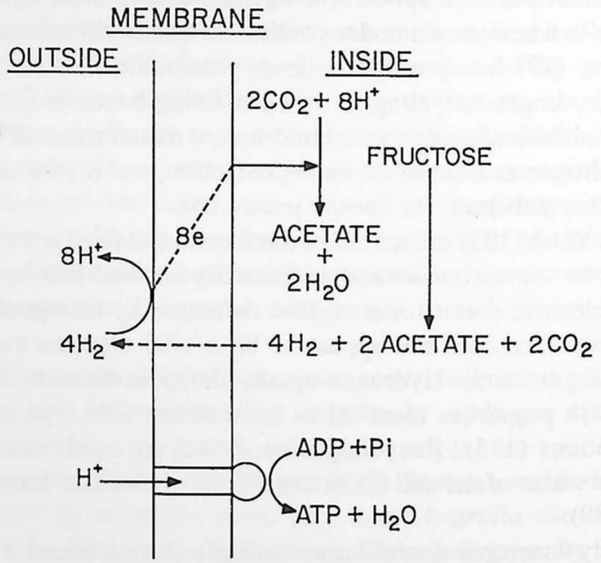


Figure 4 Postulated scheme for hydrogen cycling in *Acetobacterium woodii* (149).

In the postulated scheme, fructose is fermented to two acetate, two carbon dioxide, and four hydrogen molecules in the cytoplasm. The hydrogen diffuses across the cytoplasmic membrane, where it is oxidized by a periplasmic hydrogenase to yield eight protons and electrons. The electrons only are transferred back across the cytoplasmic membrane, where they are utilized to reduce carbon dioxide to acetate with the consumption of eight protons. As in the case of *Desulfovibrio,* this results in the transfer of eight protons across the membrane without direct coupling to electron transfer. The protons can then be utilized for transport or the generation of ATP by a reversible ATPase.

These bioenergetics proposals are in general agreement with growth yields; however, little is known about the localization of specific enzymes and electron-transfer proteins. Hydrogenase is present in many of these acetogenic bacteria: *Clostridium aceticum, A. woodii* (32), and *C. thermoaceticum* (50). *A. woodii,* like *Desulfovibrio,* accumulates low levels of hydrogen in culture headspace during heterotrophic growth and contains high levels of hydrogenase under heterotrophic or autotrophic growth conditions (32). Cultures of *A. woodii* growing heterotrophically and deprived of nickel evolve large amounts of hydrogen (48). The affected enzyme could be an uptake (NiFe) hydrogenase or carbon monoxide dehydrogenase (51).

This could be interpreted to mean that hydrogen is an intermediate between oxidations and reductions or that electron flow is simply shunted toward hydrogenase. As with *D. gigas,* heterotrophic growth of *A. woodii* is completely suppressed by an atmosphere of hydrogen, indicating an important rule for hydrogen in this fermentation. These characteristics are in line with those expected for hydrogen cycling in the case of *A. woodii;* however, another homoacetogen, *C. aceticum,* appears to be different in that growth is not inhibited by hydrogen, and hydrogenase activity is stimulated ten-fold by growth on hydrogen and carbon dioxide. The concept of hydrogen cycling offers a simple framework for understanding the bioenergetics and biochemistry of certain anaerobic bacteria. It does not necessarily require a complex electron-transfer chain and offers an explanation for multiple forms of electron transfer proteins (53), anomalous growth yields (16, 78), compartmentalization of proteins (75) and the importance of hydrogen in anaerobic fermentations (202). Hydrogen cycling may be limited to the SRB, but fragmentary data suggest that could occur in a number of other anaerobic bacteria.

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Literature Cited

1. Abram, J. W., Nedwell, D. B. 1978. *Arch. Microbiol.* 117:89–92
2. Abram, J. W., Nedwell, D. B. 1978. *Arch. Microbiol.* 117:93–97
3. Adams, M., Mortenson, L. E., Chen, J. S. 1981. *Biochim. Biophys. Acta.* 594: 105–76
4. Adman, E. T., Sieker, L. G., Jensen, L. H., Bruschi, M., LeGall, J. 1977. *J. Mol. Biol.* 112:113–20
5. Akagi, J. M. 1967. *J. Biol. Chem.* 242: 2478–83
6. Akagi, J. M. 1981. In *Biology of Inorganic Nitrogen and Sulfur*, ed. H. Bothe, A. Trebst, pp. 169–77. Berlin: Springer-Verlag
7. Akagi, J. M., Campbell, L. L. 1961. *J. Bacteriol.* 82:927–32
8. Akelagawa, K. J., Kobayashi, K., Ishimoto, M. 1983. *J. Biochem.* 93:755–62
9. Albracht, S. P. J., Graf, E. G., Thauer, R. K. 1982. *FEBS Lett.* 140:311–13
10. Albracht, S. P. J., Kalkman, M. L., Slater, E. C. 1983. *Biochim. Biophys. Acta* 724: 309–16
11. Almassy, R. S., Dickerson, R. E. 1978. *Proc. Natl. Acad. Sci. USA* 75:2674–78
12. Ambler, R. P. 1968. *Biochem. J.* 107:47
13. Ambler, R. P. 1973. *System Zool.* 22:554–65
14. Ambler, R. P., Bruschi, M., LeGall, J. 1969. *FEBS Lett.* 5:115–17
15. Ambler, R. P., Bruschi, M., LeGall, J. 1971. *FEBS Lett.* 18:347
16. Andressen, J. R., Schaupp, A., Neurauter, C., Brown, A., Ljungdahl, L. G. 1973. *J. Bacteriol.* 114:743–51
17. Badziong, W., Thauer, R. K. 1978. *Arch. Microbiol.* 117:209–14
18. Badziong, W., Thauer, R. K. 1980. *Arch. Microbiol.* 125:167–84
19. Balch, W. E., Schoberth, S., Tanner, R. S., Wolfe, R. S. 1977. *Int. J. Syst. Bacteriol.* 27:355–61
20. Banat, I. M., Nedwell, D. B., Balba, M. T. 1983. *J. Gen. Microbiol.* 129:123–29
21. Barton, L. L., Peck, H. D. Jr. 1970. *Bacteriol. Proc.* p. 134
22. Barton, L. L., LeGall, J., Peck, H. D. Jr. 1970. *Biochem. Biophys. Res. Commun.* 41:1036
23. Barton, L. L., LeGall, J., Peck, H. D. Jr. 1972. In *Horizons in Bioenergetics*, ed. H. Gest, pp. 33–51. New York: A. San Pietro Academic Press
24. Barton, L. L., Legall, J., Odom, J. M., Peck, H. D. Jr. 1983. *J. Bacteriol.* 153:867–71
25. Beinert, H., Thompson, A. J. 1983. *Arch. Biochem. Biophys.* 222:333–61
26. Bell, G. R., LeGall, J., Peck, H. D. Jr. 1974. *J. Bacteriol.* 120:994–97
27. Bell, G. R., Lee, J. P., Peck, H. D. Jr., LeGall, J. 1978. *Biochimie* 60:315–20
28. Berlier, Y., Fauque, G., Lespinat, P. A. 1982. *FEBS Lett.* 140:185–88
29. Biebl, H., Pfennig, N. 1977. *Arch. Microbiol.* 112:115–17
30. Boone, D. R., Bryant, M. P. 1980. *Appl. Environ. Microbiol.* 40:626–32
31. Brandis, A., Thauer, R. K. 1981. *J. Gen. Microbiol.* 126:249–52
32. Braun, K., Gottschalk, G. 1981. *Arch. Microbiol.* 128:294–98
33. Bruschi, M. 1979. *Biochem. Biophys. Res. Commun.* 91:623–28
34. Bruschi, M., Hatchikian, E. C., LeGall, J., Dubourdieu, M. 1969. *Bull. Soc. Fr. Physiol. Veg.* 15:381–90
35. Bruschi, M., LeGall, J. 1972. *Biochim. Biophys. Acta* 271:48–60
36. Bruschi, M., LeGall, J. 1976. In *Iron and Copper Proteins*, ed. K. T. Yasunobu, M. E. Mower, O. Hayaishi, p. 57–67. New York: Plenum

36a. Bruschi, M., Hatchikian, E. C. 1982. *Biochimie* 64:503–7

37. Bruschi, M., Hatchikian, E. C., LeGall, J., Moura, J. J. G., Xavier, A. V. 1976. *Biochim. Biophys. Acta* 449:275–84
38. Bruschi, M., Hatchikian, E. C., Golouleua, L. A., LeGall, J. 1977. *J. Bacteriol.* 129:30–38
39. Bryant, M. P., Campbell, L. L., Reddy, C. A., Crabill, M. R. 1977. *Appl. Environ. Microbiol.* 33:1162–69
40. Cammack, R., Rao, K. K., Hall, D. O., Moura, J. J. G., Xavier, A. V., et al. 1977. *Biochim. Biophys. Acta* 490: 311–21
41. Cammack, R., Patil, D., Aguirre, R., Hatchikian, E. C. 1982. *FEBS Lett.* 142:289–92
42. Cammack, R., Fauque, G., Moura, J. J. G., LeGall, J. 1984. *Biochim. Biophys. Acta* 784:68–74
43. Campbell, L. L., Postgate, J. R. 1965. *Bacteriol. Rev.* 29:359–63
44. Cappenberg, T. E. 1974. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 40: 285–95
45. Chen, J.-S., Mortenson, L. E., Palmer, G. 1976. See Ref. 36, pp. 68–81.

45a. Colbean, A., Vignais, P. M. 1981. *Biochim. Biophys. Acta* 662:271–84

46. DerVartanian, D. V., LeGall, J. 1974. *Biochim. Biophys. Acta* 346:79–99
47. DerVartanian, D. V., Xavier, A. V., LeGall, J. 1978. *Biochimie* 60:312–25
48. Diekert, G., Ritter, M. 1982. *J. Bacteriol.* 151:1043–45

49. Dixon, R. O. D. 1978. *Biochimie* 60: 233–36
50. Drake, H. L. 1982. *J. Bacteriol.* 150: 702–9
51. Drake, H. L., Hu, S. I., Wood, H. G. 1980. *J. Biol. Chem.* 225:7174–80
52. Dubourdieu, M., LeGall, J., Fox, J. L. 1973. *Biochem. Biophys. Res. Commun.* 52:1418–25
53. Elliott, J. I., Ljungdahl, L. G. 1982. *J. Bacteriol.* 151:328–33
54. Fauque, G. D., Herre, O., LeGall, J. 1979. *Arch. Microbiol.* 121:261–64
55. Fauque, G. D., Bruschi, M., LeGall, J. 1979. *Biochem. Biophys. Res. Commun.* 86:1020–29
56. Fauque, G. D., Barton, L. L., LeGall, J. 1980. In *Sulphur in Biology (Ciba Found. Symp. No. 72 New Series)*, pp. 71–86. Amsterdam: Elsevier
57. Favaudon, V., LeGall, J., Lhoste, J. M. 1976. In *Flavins and Flavoproteins*, ed. T. P. Singer, pp. 434–38. New York: Elsevier
58. Glick, B. R., Martin, W. G., Martin, S. M. 1980. *Can. J. Microbiol.* 26:1214–23
59. Grande, H. J., Dunham, W. R., Averill, B., Van Dijk, C., Sands, R. H. 1983. *Eur. J. Biochem.* 136:201–7
60. Guarraia, L. J., Peck, H. D. Jr. 1971. *J. Bacteriol.* 106:890–95
61. Guerlesquin, F., Bruschi, M., Bovier-Lapierre, G., Fauque, G. D. 1980. *Biochim. Biophys. Acta* 626:127–35
62. Guerlesquin, F., Bovier-Lapierre, G., Bruschi, M. 1982. *Biochem. Biophys. Res. Commun.* 105:530–38
63. Guerlesquin, F., Moura, J. J. G., Cammack, R. 1982. *Biochim. Biophys. Acta* 679:422–27
64. Hansan, S. M., Hall, J. B. 1975. *J. Gen. Microbiol.* 87:120–28
65. Haschke, R. H., Campbell, L. L. 1971. *J. Bacteriol.* 105:249–58
66. Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J. P., et al. 1979. *Nature* 282:806–10
67. Hatchikian, E. C. 1975. *Arch. Microbiol.* 105:249–56
68. Hatchikian, E. C. 1981. *Biochem. Biophys. Commun.* 103:521–30
69. Hatchikian, E. C., LeGall, J. 1972. *Biochim. Biophys. Acta* 267:479–84
70. Hatchikian, E. C., LeGall, J., Bruschi, M., Dubourdieu, M. 1972. *Biochim. Biophys. Acta* 258:701–8
71. Hatchikian, E. C., Chaigneau, M., LeGall, J. 1976. In *Microbial Production and Utilization of Gases*, ed. H. G. Schlegel, G. Gottschalk, N. Pfennig, pp. 109–18. Gottingen: Goltze
72. Hatchikian, E. C., Bruschi, M., LeGall, J. 1978. In *Hydrogenases: Their Catalytic Activity, Structure and Function*, ed. H. G. Schlegel, K. Schneider, pp. 93–106. Gottingen: Goltze
73. Hatchikian, E. C., Jones, H. E., Bruschi, M. 1979. *Biochim. Biophys. Acta* 548:471–83
74. Hatchikian, E. C., Bruschi, M. 1981. *Biochim. Biophys. Acta* 634:41–51
75. Henderson, C. 1980. *J. Gen. Microbiol.* 119:481–91
76. Higuchi, Y., Bando, S., Kusunoki, M., Matsuura, Y., Yasuoka, N., et al. 1981. *J. Biochem.* 89:1659–62
77. Higuchi, Y., Kusunoki, M., Matsura, Y., Yasuoka, N., Kakudo, M. 1984. *J. Mol. Biol.* 172:109–40
78. Hobson, P. N. 1965. *J. Gen. Microbiol.* 38:107–80
79. Huynh, B. H., Moura, J. J. G., Moura, I., Kent, T. A., LeGall, J., et al. 1980. *J. Biol. Chem.* 255:3242–44
80. Huynh, B. H., Czechowski, M. H., Kruger, H. J., DerVartanian, D. V., Peck, H. D. Jr., et al. 1984. *Proc. Natl. Acad. Sci. USA*. 81:in press
81. Irie, K., Kobayashi, K., Kobayashi, M., Ishimoto, M. 1973. *J. Biochem.* 73:353–66
82. Ishimoto, M., Koyama, J., Nagi, Y. 1954. *J. Biochem.* 41:763–70
83. Ishimoto, M., Kondo, Y., Kameyama, T., Yagi, T. Shiraki, M. 1958. *Proc. Int. Symp. Enzy. Chem.* pp. 229–34. Tokyo: Marzen
84. Ishimoto, M., Yagil, T. 1961. *J. Biochem.* 49:103–9
85. Johnson, M. K., Czernuszewicz, R. S., Spiro, T. G., Fee, J. A., Sweeney, W. V. 1983. *J. Am. Chem. Soc.* 105:6671–78
86. Johnson, M. K., Czernuszewicz, R. S., Spiro, T. G., Ramsay, R. R., Singer, T. P. 1983. *J. Biol. Chem.* 258:12771–74
87. Jones, H. E. 1972. *Arch. Microbiol.* 84:207–24
88. Kamen, M. D. 1983. *Fed. Proc.* 42: 2815–24
89. Kelley, B. C., Meyer, C. M., Gandy, C., Vignais, P. M. 1977. *FEBS Lett.* 81: 281–85
90. Kent, T. A., Dreyer, J. L., Emptage, M. H., Moura, I., Moura, J. J. G., et al. 1981. In *Symp. on Interactions Between Iron Proteins in Oxygen and Electron Transport*, ed. G. Ho. Amsterdam: Elsevier
91. Kent, T. A., Moura, I., Moura, J. J. G., Lipscomb, J. D., Huynh, B. H., et al. 1981. *FEBS Lett.* 138:55–60
92. Kent, T. A., Dreyer, J. L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., et al. 1982. *Proc. Natl. Acad. Sci. USA* 79:1096–100
93. Khosrovi, B., MacPherson, R., Miller, J. A. 1971. *Arch. Microbiol.* 80:324–37
94. Kimura, K., Suzuki, A., Inokuchi, H.,

Yagi, T. 1979. *Biochim. Biophys. Acta* 567:95–105
95. Knight, E. Jr., Hardy, R. W. F. 1966. *J. Biol. Chem.* 241:2752–55
96. Kobayashi, K., Hasegawa, H., Takagi, M., Ishimoto, M. 1982. *FEBS Lett.* 142:235–37
97. Kobayashi, K., Skyring, G. W. 1982. *J. Gen. Appl. Microbiol.* 28:45–54
98. Kristjansson, J. K., Schonheit, P., Thauer, R. K. 1982. *Arch. Microbiol.* 131:278–82
99. Kroger, A. 1978. *Biochim. Biophys. Acta* 505:129–45
100. Kröger, A. 1980. In *Diversity of Bacterial Respiratory Systems*, ed. C. J. Knowles, 2:1–17. Boca Raton, FL: CRC Press
101. Kröger, A., Dorrer, E., Winkler, E. 1980. *Biochim. Biophys. Acta* 589:118–36
102. Kröger, A., Winkler, E. 1981. *Arch. Microbiol.* 129:100–4
103. Kruger, H. J., Huynh, B. H., Ljungdahl, P. O., Xavier, A. V., DerVartanian, D. V., et al. 1982. *J. Biol. Chem.* 275:14620–23
104. Laishley, E. J., Travis, J., Peck, H. D. Jr. 1969. *J. Bacteriol.* 98:302–5
105. Lalla-Maharajh, W. V., Hall, D. O., Cammack, R., Rao, K. K., LeGall, J. 1983. *Biochem. J.* 209:445–54
106. Lancaster, R. 1982. *Science* 216:1324–25
107. Lee, J. P., Peck, H. D. Jr. 1971. *Biochem. Biophys. Res. Commun.* 45: 583–89
108. Lee, J. P., Yi, C. S., LeGall, J., Peck, H. D. Jr. 1973. *J. Bacteriol.* 115:453–55
109. Lee, J. P., LeGall, J., Peck, H. D. Jr. 1973. *J. Bacteriol.* 115:529–42
110. LeGall, J., Mazza, G., Dragoni, N. 1965. *Biochim. Biophys. Acta* 56:385–87
111. LeGall, J., Dragoni, N. 1966. *Biochem. Biophys. Res. Commun.* 23:145–49
112. LeGall, J., Bruschi, M. 1968. In *Structure and Function of Cytochromes*, ed. K. Okunuki, M. D. Kamen, I. Sekusu, pp. 467–70. College Park, MD: Maryland Univ. Press
113. LeGall, J., Bruschi-Heriand, M., DerVartanian, D. V. 1971. *Biochim. Biophys. Acta* 234:499–512
114. LeGall, J., DerVartanian, D. V., Spilker, E., Lee, J. P., Peck, H. D. Jr. 1971. *Biochm. Biophys. Acta* 234:525–30
115. LeGall, J., Postgate, J. R. 1973. *Adv. Microb. Physiol.* 10:81–133
116. LeGall, J., DerVartanian, D. V., Peck, H. D. Jr. 1979. *Curr. Top. Bioenergetics* 9:237–65
117. LeGall, J., Moura, J. J. G., Peck, H. D. Jr., Xavier, A. V. 1982. In *Iron Sulfur Proteins*, ed. T. G. Spiro, 4:177–248
118. LeGall, J., Ljungdahl, P. O., Moura, I., Peck, H. D. Jr., Xavier, A. V., et al. 1982. *Biochem. Biophys. Res. Commun.* 106:610–16
119. Liu, C. L. 1981. Dissertation, Univ. of Ga.
120. Liu, C. L., Peck, H. D. Jr. 1981. *J. Bacteriol.* 145:966–73
121. Liu, M. C., Peck, H. D. Jr. 1981. *J. Biol. Chem.* 256:13159–64
122. Liu, M. C., Peck, H. D. Jr., Moural, J. J. G., Moura, I., Xavier, A. V., 1984. *J. Bacteriol.* Submitted for publication
123. Lovley, D. R., Dwyer, D., Klug, M. J. 1982. *Appl. Environ. Microbiol.* 43: 1373–79
124. Magee, F. L. Jr., Ensley, B. D., Barton, L. L. 1978. *Arch. Microbiol.* 117:21–26
124a. Makano, K., Kikumoto, Y., Yagi, T. 1983. *J. Biol. Chem.* 258:12409–12
125. Martin, S. M., Glick, B. R., Martin, W. G. 1980. *Can. J. Microbiol.* 26:1209–13
126. Mayhew, S. G., Ludwig, M. L. 1975. In *The Enzymes*, ed. P. D. Boyer, 12:57–118. New York: Academic 3rd Ed.
127. Mayhew, S. G., van Dijk, G., van der Westen, H. M. 1978. In *Hydrogenases: Their Catalytic Activity, Structure, and Function*, ed. H. G. Schelgel, K. Schneider, pp. 125–40, Gottingen: Goltze
128. McInerney, M. J., Bryant, M. P., Pfennig, N. 1979. *Arch. Microbiol.* 122:129–35
129. Meyer, T. E., Bartsch, R. G., Kamen, M. D. 1971. *Biochim. Biophys. Acta* 245:453–64
130. Miller, J. D. A., Saleh, A. M. 1964. *J. Gen. Microbiol.* 37:419–23
131. Moura, I., Bruschi, M., LeGall, J., Moura, J. J. G., Xavier, A. V. 1977. *Biochem. Biophys. Res. Commun.* 75: 1037–44
132. Moura, J. J. G., Xavier, A. V., Bruschi, M., LeGall, J., Hall, D. O., et al. 1976. *Biochem. Biophys. Res. Commun.* 72: 782–89
133. Moura, J. J. G., Xavier, A. V., Hatchikian, E. C., LeGall, J. *FEBS Lett.* 89: 177–79
134. Moura, J. J. G., Moura, I., Bruschi, M., LeGall, J., Xavier, A. V., 1980. *Biochem. Biophys. Res. Commun.* 92: 962–70
135. Moura, J. J. G., Santos, H., Moura, I., LeGall, J., Moore, G. R., et al. 1982. *Eur. J. Biochem.* 127:151–55
136. Moura, J. J. G., Moura, I., Huynh, B. H., Kruger, H. J., Teixeira, M., et al. 1982. *Biochem. Biophys. Res. Commun.* 108:1388–93
137. Murphy, M. J., Siegel, L. M. 1973. *J. Biol. Chem.* 248:6911–19

138. Odom, J. M., Peck, H. D. Jr. 1981. *J. Bacteriol.* 147:161–69
139. Odom, J. M., Peck, H. D. Jr. 1981. *FEMS Lett.* 12:47–50
140. Ogata, M., Arihara, K., Yagi, T. 1981. *J. Biochem.* 89:1423–31
141. Oremland, R., Polcin, S. 1982. *Appl. Environ. Microbiol.* 44:1207–76
142. Payne, W. J., Grant, M. A. 1982. *Appl. Environ. Microbiol.* 43:727–30
143. Peck, H. D. Jr. 1959. *Proc. Natl. Acad. Sci. USA* 45:701–8
144. Peck, H. D. Jr. 1960. *J. Biol. Chem.* 235:2734–38
145. Peck, H. D. Jr., 1962. *Bacteriol. Rev.* 26:67–94
146. Peck, H. D. Jr. 1966. In *Lectures on Theoretical and Applied Aspects of Modern Microbiology*, pp. 1–22. Univ. Maryland: College Park Press
147. Peck, H. D. Jr. 1966. *Biochem. Biophys. Res. Commun.* 22:112–18
148. Peck, H. D. Jr. 1982. *Philos. Trans. Roy. Soc. London Ser. B* 298:443–66
149. Peck, H. D. Jr., Odom, M. J. 1984. In *Microbial Mats: Stromalites*, ed. H. O. Halvorson, Y. Cohen, pp. 215–43. New York: Liss
150. Pfennig, N., Biebl, H. 1976. *Arch. Microbiol.* 110:3–12
151. Pfennig, N., Widdel, F., Truper, H. G. 1981. In *The Prokaryotes*, ed. M. P. Starr, H. Solp, H. G. Truper, A. Balows, H. G. Schlegel, 1:926–40
152. Petitdemange, H., Harczak, R., Gay, R. 1979. *FEMS Lett.* 5:291–94
153. Postgate, J. R. 1956. *J. Gen. Microbiol.* 14:545–72
154. Postgate, J. R. 1959. *Nature* 183:481–82
155. Postgate, J. R. 1979. *The Sulphate-Reducing Bacteria*, Cambridge, UK: Cambridge Univ. Press
156. Postgate, S. R., Campbell, L. L. 1966. *Bacterial Rev.* 30:432–38
157. Reddy, C. A., Peck, H. D. Jr. 1978. *J. Bacteriol.* 134:982–91
158. Robinson, J. A., Tiedje, J. M. 1984. *Arch. Microbiol.* In press
159. Rozanova, E. P., Nazina, T. N. 1976. *Microbiologiya* 45:825–30
160. Sadana, J. C., Jagannathan, V. 1956. *Biochim. Biophys. Acta* 19:440–52
161. Sadana, J. C., Rittenberg, D. 1963. *Proc. Natl. Acad. Sci. USA* 50:900–4
162. Santos, H., Moura, J. J. G., Moura, I., LeGall, J., Xavier, A. V. 1984. *Eur. J. Biochem.* In press
163. Senez, J. C. 1959. *Bacterial Rev.* 26:95–107
163a. Sieker, L. C., Jensen, L. H., Pickril, B. C., LeGall, J. 1983. *J. Mol. Biol.* 171:101–3
164. Silhavy, T. J., Benson, S. A., Emr, S. D. 1983. *Microbiol. Rev.* 47:313–44
165. Smith, A. K., Emery, T. 1982. *Biochem. Biophys. Acta* 719:606–11
166. Smith, L. A., Hill, S., Hates, M. G. 1976. *Nature* 262:209–10
167. Stams, A. J. N., Hansen, T. A. 1982. *FEMS. Lett.* 13:389–94
168. Steenkamp, D. J., Peck, H. D. Jr. 1980. *Biochem. Biophys. Res. Commun.* 94:41–48
169. Steenkamp, D. J., Peck, H. D. Jr. 1981. *J. Biol. Chem.* 256:5450–58
170. Sokol, W. F., Evans, D. H., Niki, K., Yagi, T. 1980. *J. Electro. Anal. Chem.* 108:107–15
171. Suh, B., Akagi, J. M. 1966. *J. Bacteriol.* 99:210–15
172. Tabushi, I., Nishiya, T., Shimomura, M., Konitake, T., Inokuchi, H., et al. 1984. *J. Am. Chem. Soc.* 106:219–25
173. Takakuwa, S., Odom, J. M., Wall, J. D. 1983. *Arch. Microbiol.* 136:20–25
174. Tanner, A. C. R., Badger, S., Lai, C. H., Listgarton, M. A., Viscouti, R. A., et al. 1981. *Int. J. Sys. Bacteriol.* 31:432–45
175. Teixeiva, M., Moura, I., Xavier, A. V., DerVartanian, D. V., LeGall, J., et al. 1983. *Eur. J. Biochem.* 130:481–84
176. Thauer, R. K. 1982. *Philos. Trans. Roy. Soc. London Ser. B* 298:467–71
177. Thauer, R. K., Badziong, W. 1980. In *Diversity of Bacterial Respiratory Systems*, ed. C. J. Knowles, 2:65–85. West Palm Beach, FL: CRC Press
178. Traoer, S. A., Hatchikian, E. C., Belaich, J. P., LeGall, J. 1981. *J. Bacteriol.* 45:191–99
179. Travis, J., Newman, D. J., LeGall, J., Peck, H. D. Jr. 1971. *Biochem. Biophys. Res. Commun.* 45:452–58
180. Trousil, E. B., Campbell, L. L. 1974. *J. Biol. Chem.* 249:386–93
181. Tsuji, K., Yagi, T. 1980. *Arch. Microbiol.* 125:35–42
182. Unden, G., Bocher, R., Knecht, J., Kröger, A. 1982. *FEBS Lett.* 145:230–34
183. van der Westen, H., Mayhew, S. G., Veeger, C. 1978. *FEBS Lett.* 86:122–26
184. van der Westen, H., Mayhew, S. G., Veeger, C. 1980. *FEBS Lett.* 7:35–39
185. van Dijk, C., Mayhew, S. G., Grande, H. J., Veeger, C. 1979. *Eur. J. Biochem.* 102:317–30
186. van Dijk, C., Grande, H. J., Mayhew, S. G., Veeger, C. 1980. *Eur. J. Biochem.* 107:251–61
187. van Dijk, C., Grande, H. J., Veeger, C. 1982. In *Photochemical, Photoelectriochemical and Photobiological Processes*, ed. D. O. Hall, W. Palz. (D)9:190–94 Dordrecht, The Netherlands: Reidel

187a. van Dijk, C., van Berkel-Arts, A., Veeger, C. 1983. *FEBS Lett.* 156:340–44
188. van Leeuwen, J. W., van Dijk, C., Grande, H. J., Veeger, C. 1982. *Eur. J. Biochem.* 127:631–37
189. Varma, A., Shonheit, P., Thauer, R. K. 1983. *Arch. Microbiol.* 136:69–73
190. Vosjan, J. H. 1970. *Antonie van Leewenhoek J. Microbiol. Serol.* 36: 584–86
191. Vosjan, J. H. 1975. *Plant and Soil* 43: 141–52
192. Walker, C. C., Yates, M. G. 1978. *Biochimie* 60:225–31
193. Watenpaugh, K. D., Sieker, L. G., Jensen, L. H., LeGall, J., Duboordieu, M. 1972. *Proc. Natl. Acad. Sci.* USA 69:3185–88
194. Wiegel, J., Ljungdahl, L. G. 1980. In *4th Symp. Technische Mikrobiologie*, ed. H. Dellweg, pp. 117–27. Berlin: Dellweg
194a. Wiegel, J. 1980. *Experientia 36*, pp. 1434–46. Basel, Switz: Birkhäuser Verlag
195. Widdel, F. 1980. Dissertation, Univ. of Gottingen, Gottingen, Fed. Rep. Ger.
196. Widdel, F., Pfennig, N. 1981. *Arch. Microbiol.* 129:395–400
197. Widdel, F., Pfennig, N. 1982. *Arch. Microbiol.* 131:360–65
198. Widdel, F., Kohring, G. W., Mayer, F. 1983. *Arch. Microbiol.* 134:286–94
199. Wilkstrom, M., Krab, K. 1979. *Biochim. Biophys. Acta* 549:177–222
200. Winfrey, M. R., Zeikus, J. G. 1977. *Appl. Environ. Microbiol.* 33:275–81
201. Winter, J. V., Wolfe, R. S. 1980. *Arch. Microbiol.* 124:73–79
202. Wolin, M. J. 1982. In *Microbial Interactions and Communities*, ed. A. T. Bull, J. H. Slater. 1:323–56. London: Academic
203. Wolin, M. J., Miller, T. L. 1982. *Fed. Proc.* 42:109–13
204. Wood, P. M. 1978. *FEBS Lett.* 95:12–17
205. Xavier, A. V., Moura, J. J. G., LeGall, J., DerVartanian, D. V. 1979. *Biochemie* 61:689–95
206. Yagi, T. 1970. *J. Biochem.* 68:649–57
207. Yagi, T. 1979. *Biochim. Biophys. Acta* 548:96–105
208. Yagi, T., Maruyama, K. 1971. *Biochim. Biophys. Acta* 243:214–24
209. Yagi, T., Endo, A., Tsuji, K. 1978. In *Hydrogenases: Their Catalytic Activity, Structure and Function*, ed. H. G. Schlegel, K. Schneider, pp. 107–24. Gottingen: Goltze
210. Yagi, T., Tsuda, M., Inokuchi, H. 1973. *J. Biochem.* 73:1069–81
211. Yagi, T., Inokuchi, H., Kimura, K. 1983. *Acc. Chem. Res.* 16:2–7
212. Zeikus, J. G., Dawson, M. A., Thompson, T. E., Ingvorsen, K., Hatchikian, E. C. 1983. *J. Gen. Microbiol.* 129: 1159–69

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