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MY KIND OF BIOLOGY

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Because the rigid format of scientific publishing renders the published product nearly sterile of the fun of doing science, I have attempted to provide here some background and perspective on the contributions to science in which my laboratory played a part.

Toward Microbiology

I was 25 years old before I saw a living bacterial cell. As an undergraduate, I had majored in biology at Bridgewater College in Virginia, a small liberal arts college. Undergraduate biology at that time was mostly descriptive, but "Doc" Jopson in the biology department insisted that I take organic chemistry

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and minor in chemistry; this advice was sound. I had been raised on the campus of a small college where my father taught religion and philosophy. He would go to the campus, present a lecture, and return home to work in his shop or garden. His summers were free of college obligations. To me in my late teens, this seemed an ideal way to spend one's life. The pay was poor, but the freedom was fantastic—especially with a three-month summer. After I had taught high school for a brief period, a friend who had just received his Master's degree in history at the University of Pennsylvania recommended Penn as a "good university"; so I hitchhiked to Philadelphia intent on getting a MS degree and then teaching in a small college. (I never reached the small college.)

In my youth, I had been fascinated by petrified bones and had thought that working in a museum or digging up petrified skeletons would be exciting. In Philadelphia, I went to the Academy of Natural Sciences, visited the paleontology research area of the museum, and talked to a curator. The experience was rather sobering. Here was an investigator in a dimly lit area working under a light bulb, surrounded by what seemed to be acres of petrified bones. I remember thinking on my way out that I would have to find something more alive than this.

A fixture at many universities are frustrated individuals who enjoy keeping graduate students in line. When I inquired at the graduate college office of the university about the possibility of doing graduate work in biology, I was told, "We don't have a department of biology. We have a department of botany and a department of zoology; now which will it be?" Off the top of my head I blurted out, "botany." "Well in that case, you must go to McFarlane Hall and talk with Dr. Schramm." Professor Schramm was a white-haired kindly man who seemed interested in me. Instead of asking what courses I had taken, he inquired as to what I knew about various subjects. One question in particular stands out in my memory: "Do you know anything about bacteriology?" I replied that I knew nothing, but that I had often thought that might be an interesting subject. So, he signed me up for general bacteriology. This was the only course I could take because I needed to find employment to support myself, and I asked him if any jobs were available in the department. A job in the herbarium involved mounting pressed flowers on sheets of paper; would I be interested? A job was a job, and since I was to be a student in botany this seemed to be an ideal way to learn the names of flowers. Besides, I could set my own hours. So my career as a graduate student in botany was launched.

I was enthusiastic, and on my way to the first class in bacteriology I wondered if I would get to see a living bacterial cell. I did; I was fascinated. As the laboratory course unfolded, the fact that one could start to grow these living cells in the afternoon and the next morning could read the results seemed incredible to me. This was my kind of biology. The course was taught

by W. G. Hutchinson, and he turned me on to bacteriology. I did well—after all I was only taking one course—and when an opening occurred for a teaching assistant in bacteriology, Professor Hutchinson invited me to assist him. I was elated and did not hesitate to leave the job of mounting flowers. It had become painfully obvious to me that something was wrong; I could not remember the names of the plants! In contrast, I had no trouble remembering the names of bacteria.

So as my second year in graduate school began, I moved to the old public health laboratory on 34th Street where Professor Hutchinson introduced me to a young assistant professor, D. J. O'Kane, who had just arrived from Cornell and was setting up his laboratory. I was to be a teaching assistant in general bacteriology and would take a new course being developed by O'Kane to give students some exposure to biochemical activities of microbes. I never had a formal course in biochemistry, and sometimes it shows.

As a teaching assistant, I soon found that a successful laboratory course was based on thorough preparation. The degree of success depended on attention to details; everything must be checked: the cultures, the media, the glassware, the incubator. Assume nothing! It was great when everything worked. I especially enjoyed being a teaching assistant with W. G. Hutchinson. However, his interests were not in the area of bacterial physiology and metabolism, an area that was on the forefront at the time and that seemed attractive to me. So O'Kane accepted me as a graduate student, one intent on getting a terminal Master of Science degree. I worked on the enzyme hippicurase, from *Streptococcus*, a hydrolytic enzyme that cleaved a peptide-like bond and was considered of interest because the mechanism of peptide bond synthesis was unknown at the time. My initial attempts at research were rather painful for my professor, I'm afraid, especially when I didn't even know how to plot the data I generated. I needed to be spoonfed. Eventually, I found that doing the experiment on my own without letting my professor know was fun. When I thought I had established a scientific fact, we would talk. I liked this system; the thrill of discovery on one's own is the best motivating force.

After receiving a MS degree, I decided to take a year off from graduate school. Graduate students were paid $950 for nine months, so it was necessary to save enough money during that period to live during the summer. I wanted to see what nonacademic life was like and try to shore up my financial condition. I accepted a position as a technician in Ruth Patrick's laboratory at the Academy of Natural Sciences. She had become interested in devising ways for assaying the toxicity of stream pollutants. This work broadened my appreciation of biology. My professors at Penn encouraged me to continue graduate studies toward a PhD degree, but before returning to the university, my life became complete; Gretka Young, who worked for the American Friends Service Committee, and I were married in September, 1950. My goal

of teaching in a small college would become untenable in the next three years as my professors made me aware that the PhD degree was a research degree, that to do research one needed proper equipment, and that adequate facilities were rarely found in a small college. I eventually would realize that I had been scientifically seduced.

Professor O'Kane offered me a choice of two thesis topics involving a new factor, the pyruvate oxidation factor, which he had worked on in the laboratory of I. C. Gunsalus at Cornell. This factor was later identified as lipoic acid. He wanted to know whether lipoic acid was involved in the oxidation of pyruvate by *Escherichia coli* and *Clostridium butyricum*. I instantly chose the clostridial system because it seemed challenging and unknown. I was able to show with treated extracts that diphosphothiamin, coenzyme A, and ferrous ions were required for the oxidation of pyruvate, but I could find no role for lipoic acid (166). Various dyes could be used as electron acceptors to bypass hydrogenase; however, I could not make progress on the natural electron acceptor. Exchange of $^{14}CO_2$ with the carboxyl group of pyruvate occurred readily (167). These experiments introduced me to the use of radioisotopes. I completed my thesis work in June 1953, a few months after our first child, Danny, was born.

The event that encouraged me to consider the possibility of an academic position in a research environment involved the preparation of my first manuscript from part of my thesis. I was apprehensive about submitting a manuscript to the *Journal of Biological Chemistry* because I lacked confidence in writing. However, I carefully patterned the manuscript in the style of the journal and gave it to my professor. He returned it and announced, "I think this is fine, Ralph; let's send it in." We did, and it was accepted! This was the first time anyone had ever expressed approval of my writing and gave me confidence that perhaps I could become successful at scientific writing. I owe much to D. J. O'Kane, who made me appreciate the importance of hard data in nailing down a concept as well as the importance of freedom in exploring and making discoveries. Later, I would use this same philosophy in running my own laboratory.

Very few academic jobs were available in the summer of 1953. I had two interviews, one at a small college, which had only a case-full of student microscopes as equipment, and one at the University of Illinois. I was hired as an instructor at Urbana by H. O. Halvorson. My case had been presented in a sufficiently positive way through the efforts of my scientific grandfather, I. C. Gunsalus (known to everyone as Gunny). The Department of Bacteriology was an exciting place; with recent appointments of Halvorson, Spiegleman, Luria, Gunsalus, and Juni, the department was considered one of the best in the country, and I was fortunate to join it. A heavy teaching load didn't leave much time for research, and my program was rather slow in evolving. Before

I started anything, I was determined to prepare a manuscript for publication from the last part of my thesis. So I closed my office door whenever I had a chance and worked on the manuscript.

This action was interpreted as inaction but led to a revelation of one of the truisms of academic life. One day there was a knock on my office door. Professor Halvorson entered, sat down, and in a very concerned manner said, "I just want to tell you one thing—you are paid to teach; you get promotions for doing research." He departed immediately, and I pondered these words of wisdom. They are as true today as they were 38 years ago.

The faculty wanted to augment their specialties with someone who had a real interest in diverse organisms and who would want to teach a van Niel–type course. Largely through persuasion by Gunny, van Niel accepted me as an observer in his course at Pacific Grove for the summer of 1954. The class was a fantastic experience that opened my eyes to a microbial world of unfamiliar organisms and made it possible for me to attempt to fill the niche for which I had been hired at Urbana. I returned to Illinois with many ideas from van Niel that, together with some from Gunny, Luria, Sherman, and myself, became an organisms course that would be taught for nearly three decades.

When I left Penn, O'Kane generously allowed me to take the pyruvate clastic system with me to serve as a basis for my research program. In response to an inquiry about equipment that I would need to get started, I had suggested to Professor Halvorson a colorimeter, a vacuum pump (with which to freeze dry cell extracts) and a Warburg apparatus. He seemed a little dismayed by this, and I thought his response was a bit curious, for the request seemed modest to me. A year later, I was told, "We have enough biochemists around here; I hired you because I thought you weren't one." The message was clear—I had better begin visibly studying unusual organisms or I did not have a future at Illinois. Having a mandate to study such organisms was great, but I also instinctively knew that to gain the respect of the scientific community I must be involved in an in-depth study of a biochemical phenomenon. Isolation and cultivation of "funny bugs" (although challenging and rewarding) alone was not enough. I knew my abilities would limit how far I could go, but I was determined to become a respectable microbiologist.

Ferredoxin

As a graduate student I poured ferredoxin down the sink for three years. Fortunately, much later one of my own graduate students was involved in its discovery. At the time of my studies, the research community knew that clostridia did not possess cytochromes. Because no other protein electron carriers were known, we assumed that the unknown electron acceptor for pyruvate oxidation would be a soluble cofactor. Formation of carbon dioxide

and acetyl phosphate from pyruvate could readily be followed in treated cell extracts upon addition of dyes, but I could find no evidence for a coenzyme that could play a role similar to nicotinamide adenine dinucleotide (NAD) or flavin adenine dinucleotide (FAD). When I accepted my first graduate student, Robert Mortlock, at Urbana, I suggested that he identify the electron acceptor and study the reversal of the reaction. He obtained convincing evidence for synthesis of pyruvate from acetate and carbon dioxide (99, 100). The nature of the electron acceptor remained obscure, however.

In the summer of 1957, an undergraduate student, Raymond Valentine, expressed an interest in learning about research with bacteria, and I suggested that he begin by helping Mortlock with studies on the pyruvate clastic reaction. Valentine was highly motivated, and the two developed an isopropanol precipitate of crude cell extract that actively decarboxylated pyruvate with methyl-viologen as electron acceptor. He realized the significance of this resolved extract and wanted to pursue study of the natural electron acceptor for a thesis.

One of the most difficult decisions in science is deciding when to quit one line of research and start a new one. There is always the possibility that one more cast might do it. I had been casting for a strike on the electron acceptor for about eight years and was eager to try something new. So, much to Valentine's disappointment, we dropped the clastic reaction and began to study anaerobic allantoin degradation. To augment Valentine's training, arrangements were made for him to spend a summer working on organic synthesis with Roe Bloom, an organic chemist with whom I was acquainted at the DuPont Company in Wilmington, Delaware. In the meantime, a paper by the DuPont nitrogen fixation group headed by Carnahan appeared. Mortenson had found that the addition of pyruvate to extracts of *Clostridium pasteurianum* greatly stimulated nitrogen fixation. We realized that the electron acceptor from pyruvate oxidation for which we had an assay might be the electron donor for nitrogenase, and Valentine communicated this to Gunny, who was a consultant to DuPont at the time. Gunny picked up the phone and arranged for Valentine to join the nitrogen fixation group for the summer. He applied the isopropanol precipitation procedure developed in Urbana to extracts of *C. pasteurianum* and produced a methyl-viologen-dependent pyruvate clastic reaction. With this assay, he began to test various nitrogenase preparations and fractions. Mortenson suggested that he should test a brown preparation stored in a certain refrigerator, a fraction prepared by Ralph Hardy before he left the company. When he opened the refrigerator, he found two bottles, each of which contained a brown fluid. The first one tested instantly denatured the proteins of the assay mixture; it turned out to be chromate cleaning solution. A sample from the second complemented the resolved enzyme preparation; the electron carrier had been located and could

donate electrons to hydrogenase. (The first name considered for the carrier was "co-hydrogenase.") Under Mortenson's leadership, the protein was fractionated, purified, and shown to be an iron-sulfur protein, a new type of electron carrier. The naming group at the DuPont Company named this new product ferredoxin.

Upon returning to Urbana, Valentine wanted to pursue ferredoxin as a thesis, but I felt that the discovery had been made in DuPont laboratories, and to continue to study the ferredoxin of a saccharolytic *Clostridium* in my laboratory would be inappropriate. However, looking for ferredoxin in other anaerobes would not be a problem. We used the finding of ferredoxin in *Micrococcus lactilyticus* as a lever with which to pry the original proprietary data on *C. pasteurianum* into publication, and the papers were published back-to-back (98, 139). In the next 10 years, hundreds of papers on this new type of iron sulfur protein electron carrier, ferredoxin, appeared. We explored several ferredoxin-dependent reactions (18, 19, 136, 137, 140, 145).

I wished to know if the pyruvate reactions in *Bacillus macerans*, one of the sugar-fermenting members of the genus *Bacillus*, were similar to those of *C. butyricum*. Raymond Hamilton (65) studied the exchange reactions of CO_2 and of formate with the carboxyl group of pyruvate and found that the CO_2 exchange reaction was similar to that in *C. butyricum*, but that a separate formate-pyruvate exchange reaction also occurred.

Methanobacillus omelianskii

In 1960, I arranged to spend my first sabbatical leave in the laboratory of Sidney Elsden at Sheffield, England. He had been a student of Marjorie Stephenson and C. B. van Niel and was interested in microbial physiology. I thought he would be an ideal sounding board for the major purpose of my sabbatical, which was to write a guide to the isolation of unusual organisms from nature, for which I had received a Guggenheim Fellowship. After about a month in the library, I realized that this project wasn't much fun, so I threw down the pencil and moved to a lab bench. We decided that methane bacteria would be a good project. H. A. Barker kindly sent us a culture of *Methanobacillus omelianskii*, which I was able to culture. Our purpose was to study the synthesis of amino acids from [^{14}C]-labeled CO_2 and acetate by this unusual anaerobe. We got off to a good start; the labeling patterns were definitive on several amino acids, and Martin Knight continued the project for his thesis (85).

I returned to Urbana convinced that *M. omelianskii* could be mass cultured, and that the time was ripe to go after the biochemistry of methanogenesis. My colleague, M. J. Wolin, in the Department of Dairy Science, was interested in this subject, so I invited him to collaborate. We hired Eileen Wolin to initiate cultures and scale them to the 3-liter–florence flask stage. They were then

brought to my laboratory where carboys were inoculated. We developed a production line so that we could harvest a carboy of cells at least three times a week, each carboy yielding enough cells for one experiment. Cells were immediately broken in a Hughes press, and a dark brown cell extract was prepared. Norman Ryckman played an essential role in growing and harvesting cells and in preparing cell extracts. Old Warburg flasks fitted with serum stoppers served as reaction vessels, and samples of the flask's atmosphere were transferred by syringe to a gas chromatograph to test for methane formation. The first extract was tested in October 1961, and for the next five months the recorder pen never moved from the baseline except to respond to a standard injection of methane. Extracts refused to oxidize ethanol or acetaldehyde and reduce CO_2 to methane under any condition. In March, out of desperation, I tipped my old friend, pyruvate, into the extract. I shall never forget the zing of the recorder as the pen soared to the top of the chart and back precisely at the time methane should elute from the column. The first formation of methane by a cell-free extract had occurred (171).

This assay allowed us to optimize the system and to show that the role of pyruvate was to provide electrons, carbon dioxide, and ATP. The discovery by Blaylock & T. Stadtman (11) that the methyl group of methylcobalamin could be reduced to methane by extracts of *Methanosarcina* in the presence of pyruvate was a major breakthrough. M. J. Wolin synthesized methylcobalamin, and we showed the ATP-dependent reduction of the methyl group to methane by extracts of *M. omelianskii* (172) and that B_{12r} was the product (173). John Wood (178) studied the reaction and showed that the cobamide derivatives, methyl-Factor B, and methyl-Factor III also were effective methyl donors for methanogenesis (180). The ferredoxin-dependent conversion of formate or acetaldehyde was worked out by Winston Brill (18, 19), and Wood (176) showed that the methyl group of methyl-tetrahydrofolate was reduced to methane. Additional studies suggested that carbon-3 of serine was a precursor of methane via conventional C_1-tetrahydrofolate intermediates (175). Wood also obtained evidence for alkylation (177) and propylation (179) of a cobamide enzyme involved in methanogenesis. Wolin (170) found that viologen dyes were potent inhibitors of methanogenesis. These experiments were exciting as we groped to figure out how extracts of *"M. omelianskii"* made methane from carbon dioxide. The laboratory of T. Stadtman was the only other group actively working on this project.

Interspecies Hydrogen Transfer

In the summer of 1965, I asked Marvin Bryant, who had recently joined the Department of Dairy Science, if he would teach me the Hungate technique. For the roll tube experiment, he suggested that we carry out an agar dilution series using *M. omelianskii* because one of his colleagues was not satisfied

that the culture met the criteria of a pure culture. We decided to use a rich medium that contained rumen fluid to encourage the growth of any contaminants. In one series of roll tubes, we added H_2 and CO_2. We picked isolated colonies back into the ethanol carbonate medium of Barker, but nothing grew—not an unusual observation when working with methanogenic bacteria at that time. Soon the summer was over, and we returned to other duties. About seven months later, Bryant found a rack of roll tubes from our experiments in the incubator. One tube contained a large isolated colony, and, when he removed the rubber stopper, he found a strong negative pressure. The organism oxidized hydrogen, and a serial dilution in agar roll tubes established that the culture was pure and that it made methane. The organism was labeled strain M.o.H. and would not grow in the ethanol medium of Barker, so Hungate suggested to Bryant that the original culture must contain a companion organism that used ethanol as a substrate, and he should go after it. After many difficulties, Bryant succeeded in isolating the S organism, which was inhibited by the hydrogen that it produced.

M. J. Wolin realized what was going on. He blew the dust from his physical chemistry book and calculated the free energy change for the oxidation of ethanol to acetate and hydrogen vs the partial pressure of hydrogen. *M. omelianskii* was a symbiotic association of two organisms. The S organism oxidized ethanol to acetate and hydrogen; the methanogen, *Methanobacterium* strain M.o.H., lowered the partial pressure of hydrogen by oxidizing it to reduce CO_2 to CH_4; this allowed the anaerobic oxidation of ethanol by the S organism to become thermodynamically favorable. Thus, interspecies hydrogen transfer (the importance of the partial pressure of hydrogen in anaerobic biodegradation), one of the first principles of anaerobic microbial ecology, had been discovered. We thought this paper would be a suitable way to honor C. B. van Niel (22).

With the discovery that *M. omelianskii* was a mixed culture, the roof of my research program more or less collapsed. Much of Winston Brill's thesis could not be published because we did not know which enzymes came from which organism. During this work, I could not figure out why such a bright, dedicated student was having so much difficulty with variability of cell extracts. I have always regretted this and feel that I should have been more astute about the culture. Much of Abdel Allam's thesis could not be published, and Richard Jackson's work with Lovenberg on the amino acid sequence of ferredoxin was in doubt concerning which organism produced the iron sulfur protein. Five years of work on methanogenesis by extracts of *M. omelianskii* needed to be reinterpreted.

The real challenge now was to develop a mass culture technique and a method for growing cells on H_2 and CO_2. I accepted this challenge, but all attempts to culture strain M.o.H. by sparging gas through a liquid medium

failed. In desperation, I developed a closed system in which a diaphram pump recirculated the gas atmosphere over heated copper and back into the culture vessel. I named this gadget the gaspirator. (Spiegleman referred to it as Wolfe's last gaspirator.) It provided a few good runs, but its performance was erratic, and it was abandoned. However, I developed a system for slowly shaking 200 ml of medium under a gas atmosphere of H_2 and CO_2 in a flask with a small continuous addition of gas (21). I was delighted that the inoculum grew when transferred to a 12-liter fermentor and could then be harvested or used to inoculate a 200-liter fermentor. Soon we had kilogram quantities of cells. This work could not have been done without Marvin Bryant, who patiently provided us with inocula of strain M.o.H. Langenberg (87) documented the electron microscopy of strain M.o.H. Anthony Roberton (109) studied the ATP requirement for methanogenesis from methylcobalamin by cell extracts of strain M.o.H. He showed that intracellular pools of ATP increased when cells were oxidizing hydrogen and carrying out methanogenesis, but decreased when hydrogen was removed (110).

Coenzyme M, the Terminal Methyl Carrier

Barry McBride arrived from the University of British Columbia, Vancouver, at a propitious time. A technology for growing cells on H_2 and CO_2 had been developed; he was able to contribute to the mass culture of cells at the 12-liter stage and was the first one to have the courage to grow a 200-liter batch of strain M.o.H on H_2 and CO_2. He discovered a new cofactor that was required for the formation of methane from the methyl group of methylcobalamin by cell extracts. Evidence suggested that this cofactor was involved in methyl transfer, so we named it coenzyme M (CoM) (94). He found a curious inhibition of methanogenesis by DDT (95). In testing various buffers to optimize the assay for CoM, he noticed that a strong garlic-like odor was produced in arsenate buffer, and we (96) documented the synthesis of dimethylarsine by cell extracts from methylcobalamin and arsenate. His work opened up a new era in the biochemistry of methanogenesis, one that we would follow for 20 years. We owe much to Barry McBride, who later pioneered use of the Frêter chamber for handling methanogens, the growth of methanogens on Petri dishes, the fluorescence of methanogen colonies, the use of the epifluorescence microscope to detect individual cells of methanogens, and the bright fluorescence of protozoa. Unfortunatcly, he did not receive proper acknowledgment for the discovery that individual cells of methanogens fluoresce.

The study of CoM was taken up by Craig Taylor, who over a five-year period purified the factor to homogeneity and determined its structure as 2-mercaptoethanesulfonic acid (128). He showed that the coenzyme was methylated on the reduced sulfur atom to form 2-(methylthio)ethanesulfonic

acid, CH_3-S-CoM. Both forms of the coenzyme were chemically synthesized. CoM was identified as the unknown growth factor required by *Methanobrevibacter ruminantium* (126). Later, William Balch (8) studied its transport. A simplified enzymatic assay was developed for the synthesis of CH_3-S-CoM with methylcobalamin as the methyl donor, and the transmethylase that catalyzed this reaction was purified (127).

Because CH_3-S-CoM served as the substrate for methane formation in the absence of methylcobalamin, we considered both methylcobalamin and the transmethylase to be outside the natural pathway of methanogenesis. Attention now became focused on the CH_3-S-CoM methylreductase reaction, and Robert Gunsalus pursued this reaction as a thesis topic (64). He collaborated with James Romesser in synthesizing a variety of CoM analogues (59). We wanted to know just how specific the coenzyme was, and of all the derivatives tested only ethyl-CoM showed a positive effect, producing ethane at 20% of the rate of methane. We had noted that certain preparations of CH_3-S-CoM would not serve as a substrate for the methylreductase reaction, and we did not understand why until bromethanesulfonate (BES) was found to be a potent inhibitor of methanogenesis (59). We then made sure that any excess BES, the starting compound for the synthesis, was removed from preparations of CH_3-S-CoM.

Archaebacteria

As a result of graduate training and exposure to ideas of the Delft school, I had become a firm believer in the unity of biochemistry for the biological world. In my own laboratory, the 1926 paper by Kluyver & Donker (84) had a special impact on students during our studies on electron transport in clostridia, especially since we felt we were a part of the family tree to Kluyver through contact with van Niel. Perhaps students today should be made more aware that they are part of a continuum—that their experiments have scientific roots! For vitamins and coenzymes, the case for biochemical unity was particularly strong.

The discovery of coenzyme M and the elucidation of its structure revealed a chance to document the distribution of a new vitamin-coenzyme relationship with a classic microbial growth-dependent assay. Not since lipoic acid had there been such an opportunity. Perhaps CoM would have a similar distribution as well as an important role in methylation reactions. However, growing a hydrogen-oxidizing methanogen using the Hungate technique was difficult. Each tube had to be opened and regassed with H_2 and CO_2 more than twice a day for 4 or 5 days. The negative pressure developed inside each tube made it especially difficult to prevent contamination by O_2 and bacteria when the stopper was removed. The single figure (126) showing that CoM was the

growth factor for *M. ruminantium* represented efforts made over a large portion of a year. Assay after assay failed in Bryant's laboratory, and finally the master himself took his spring break to generate the data. I thought there must be a better way. I suggested to a new student, William Balch, who was tooling up to document the distribution of CoM in the biological world for part of his thesis work, that to avoid the pitfalls of this assay (which only an expert like Bryant could handle) we should try to develop a system in which cells could be grown in a pressurized atmosphere.

Macy, Snellen, & Hungate (93) had pioneered the use of syringes for the transfer of oxygen-sensitive bacteria in the Hungate technique. Miller & Wolin (97) extended the use of syringes to the inoculation of media contained in a standard serum bottle with an aluminum-crimped seal. In addition, they had the manufacturer put the serum vial top on standard culture tubes. This procedure worked well for fermentative microbes, which produced gas, but the seal was not designed to hold for organisms such as methanogens that created a negative pressure during growth. Balch replaced the standard serum rubber seal with a solid rubber stopper, and pressurized the H_2 and CO_2 atmosphere above the medium to 2 atm. More carbonate was added to the medium to increase the buffering capacity. The medium was inoculated by use of a syringe, and the atmosphere could be repressurized aseptically so that a standard growth curve was produced. Contamination was no longer a problem. A special stopper with a lip was designed, and Bellco Glass agreed to market it (6). The Balch modification of the procedures of Macy & Hungate and Miller & Wolin became standard procedure for the field (3), and in the hands of Karl Stetter proved to be equally valuable for isolation of extremely thermoacidiophilic archaebacteria (163).

In Balch's hands, the CoM growth-dependent assay became routine and could easily detect 10 pmol. With this sensitive assay, over the next two years he tested animal tissues of all types as well as a wide range of plants and microbes (7). The answer was clear-cut: the new vitamin-coenzyme was present only in methanogens! I was disappointed; not only had the unity of biochemistry thesis let me down, but it appeared as if we had spent two years on a fruitless endeavor. However, Balch had thoroughly documented a fact that later would become important and had perfected the new system for growing methanogens in a pressurized atmosphere. His expertise would soon reap unexpected collaborative discoveries that otherwise might not have been possible. Carl Woese and I had been discussing a proposed analysis of the 16S rRNA of methanogens. Because a sealed rcliable growth procedure had been developed, we entered into a collaboration in which the ability to label the nucleic acids of methanogens with high ^{32}P-specific activity was the limiting parameter.

Woese had found that growth of slow-growing organisms could easily cease because of radiation damage before sufficient label could be in-

corporated. Balch, with his pressurized atmosphere technique, had the key to success in coaxing each methanogen to take up sufficient label before dying. He developed a close working relationship with George Fox in Woese's laboratory, and each methanogen was a special challenge. When I asked Woese about the results of the first attempt to label the 16S rRNA of a methanogen, he replied that something had gone wrong with the extraction—perhaps they had isolated the wrong RNA. The experiment was repeated with special care, and this time Carl's voice was full of disbelief when he said, "Wolfe, these things aren't even bacteria."

When we started these experiments in 1976, Woese's laboratory had previously analyzed the T_1 endonuclease–generated 16S rRNA oligonucleotides of 60 species of microbes representing a wide variety. Against this wealth of background information, the data from the methanogens were perceived to be clearly different. Analysis of other methanogenic species supported the conclusion that methanogens were only distantly related to typical bacteria with regard to their 16S rRNA (54). And now the curious coenzymes assumed special importance. CoM had been shown to be unique to methanogens, and Dudley Eirich had just finished working out the structure of the first natural deazaflavin, the unique coenzyme F_{420}.

So the concept that methanogens represented an ancient divergence in evolution was initially a two legged stool—one leg supporting the concept was the 16S rRNA oligonucleotide data and the other was the "crazy coenzymes." We needed more legs on the stool, and I thought that if methanogens were this different in these areas, they also should exhibit other unusual properties. I wrote to Otto Kandler in Munich asking if he would be interested in examining cell walls of methanogens that we could send to him. He was enthusiastic for the project, and his laboratory soon showed that, indeed, *Methanobacterium* species not only lacked the typical peptiodglycan cell wall structure, which was known to be a characteristic of all bacteria except the mycoplasma, but possessed instead a pseudomurein; other methanogens had no peptidogylycan at all. We now had a third leg on the stool supporting the concept that methanogens were only distantly related to typical bacteria (4). Soon, many legs would support the concept, but all of these data only made sense when Carl Woese proposed that methanogens, extreme halophiles, and certain thermoacidophiles belonged to a distinct phylogeny, the archaebacteria (161), now known as the Archaea (162).

Many researchers were sceptical about this concept and about the the use of 16S rRNA to establish a phylogeny for the microbial world. We were fortunate at Illinois to have Marvin Bryant, who had written the section on methanogenic bacteria for *Bergey's Manual of Determinative Bacteriology*, as a colleague. The acceptance of the methanogens as a distinct group was controversial; the popular press did not help by calling them a "third form of life."

We decided to prepare an article for *Microbiological Reviews* in which all evidence that supported the uniqueness of the methanogens could be evaluated readily by the scientific community (3). The backbone of the paper was the oligonucleotide analysis prepared in Woese's laboratory. This article gained the the respect of the scientific community. Working with Carl Woese was a unique experience, one of the high points in my scientific life. I gained respect and admiration for him, one of the most dedicated scientists I have known.

Coenzyme F_{420}, the Deazaflavin

While fractionating cell extracts of strain M.o.H., Paul Cheeseman observed an abundant yellow compound that exhibited a bright blue-green fluorescence under UV light. He purified this compound to homogeniety and Ann Toms Wood continued these studies (27). The compound was named factor-420 (F_{420}) because of the strong absorption maximum at 420 nm. Godfried Vogels chose to study the fluorescence spectra and other properties of F_{420} during a sabbatical leave at Urbana. In Bryant's laboratory, extracts of *M. ruminantium* grown on formate as substrate exhibited both formic dehydrogenase and hydrogenase activity. During fractionation, NADP reduction by hydrogenase was found to require an unknown factor. A sample of our preparation of F_{420} substituted; the K_m for F_{420} was 5×10^{-6} M (133). A similar story developed for the F_{420}-dependent reduction of NADP by formic dehydrogenase in which the enzyme also showed a high specificity for F_{420} (132). These papers documented the role of F_{420} as an electron carrier in methanogens.

I suggested to Dudley Eirich that the structure of F_{420} would make a good thesis topic. After a course in organic qualitative analysis, he began to analyze hydrolytic fragments of F_{420}, and after a five-year dedicated effort, he had a structure in hand (36). The kind interest of K. L. Rinehart, Jr. as well as his willingness to be a sounding board were essential to the success of the project. The coenzyme is the N-(N-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5' phosphate. The distribution of F_{420} and properties of its hydrolytic fragments were published (37). F_{420} was the first naturally occurring deazaflavin to be described. In contrast to CoM, it is found in certain organisms other than methanogens, although the quantities in methanogens may be vastly greater.

Factor 342, Methanopterin, the Coenzyme of Methyl Transfer

When fractionating cell extracts by column chromatography, Robert Gunsalus noted a compound that fluoresced bright blue under UV light (63). This compound was named factor 342 (F_{342}) because of its absorption at 342 nm. No role was found for the factor, but Vogel's laboratory showed that it was a pterin derivative. James Romesser started another line of investigation when

he began to study methanogenesis from formaldehyde. Hydroxymethyl-CoM, which was readily synthesized from formaldehyde and HS-CoM, was proposed as an intermediate in methanogenesis (112). The compound was found to hydrolyze in water to yield HS-CoM and formaldehyde, but the rate was much slower than the rate of methanogenesis from hydroxymethyl-CoM catalyzed by cell extracts, so we believed at the time that it was an intermediate. Later, Jorge Escalante showed that, because formaldehyde was removed from solution by methanogenesis, the equilibrium was drastically displaced toward hydrolysis; hydroxymethyl-CoM was not an intermediate in methanogenesis (48).

However, Escalante discovered a factor that was required for methanogenesis from formaldehyde that was named the formaldehyde activation factor (FAF). The factor was purified anaerobically to homogeniety; in the reduced form it had a molecular weight of 776, whereas that of the oxidized form was 772 (45, 46). The factor had properties very similar to methanopterin described by Vogel's laboratory (83), and Escalante showed that FAF was tetrahydromethanopterin (45, 46, 49). Escalante documented the role of the methenyl, methylene, and methyl derivatives of tetrahydromethanopterin in methanogenesis (47). In Neijmegen, the structure of the yellow fluorescent compound of Daniels' was firmly established to be methenyl-tetrahydromethanopterin (146). Mark Donnelly (33, 34) documented the role of 5-formyl-tetrahydromethanopterin as a C_1 intermediate in methanogenesis. So the blue fluorescent compound F_{342} led to the discovery of the central C_1 carrier of methanogenesis.

Coenzyme F_{430}, the First Nickel Tetrapyrrole

In 1977, we sent some frozen cells of *Methanobacterium* to Gregory Ferry, who was pursuing postdoctoral studies with Harry Peck at Athens, Georgia. Somehow an extract of these cells ended up in the hands of Jean LeGall. He communicated to us the presence of a yellow nonfluorescent compound in these extracts and provided us with a sample, stating that he had a hunch the compound might be a cobamide derivative. Robert Gunsalus repeated the isolation, but the compound was unintercsting to me: when added to extracts, it neither inhibited nor stimulated methanogenesis. I thought it might be a carotenoid. We namcd it factor 430 (F_{430}) because of its dramatic absorption at 430 nm (63). Later, William Whitman submitted F_{430} for neutron activation analysis, and the results suggested that the compound containcd one nickel atom per molecule (152). This finding was of considerable interest, and we were pondering experiments when we received a note from Rolf Thauer saying that F_{430} contained nickel and that the incorporation of ^{63}Ni into F_{430} during growth was documented (29). In addition, his laboratory produced convincing evidence for the incorporation of δ-aminolevulinic acid into F_{430}

(28). This work stimulated Eschenmoser's group to document the structure of F_{430} as a pentaacid tetrahydrocorphin (92, 107). But we still had no role for F_{430} until William Ellefson observed that homogeneous CH_3-S-CoM methylreductase was yellow and had an absorption maximum at 425 nm. Ellefson & Whitman grew cells with ^{63}Ni in the medium, and when the homogeneous radioactive methylreductase was allowed to react with specific antibodies in an immunodiffusion plate, the precipitated protein-antibody band was labeled with ^{63}Ni. The methylreductase contained two molecules of F_{430} per M_r 300,000 protein (38). So F_{430}, the compound that wouldn't do anything, now is considered to be at the very heart of methanogenesis. Recently, Karl Olson collaborated with Michael Summers in Baltimore on the 2D and 3D NMR analysis of native F_{430}, the 12-13 dieprimer, and the reduced derivative F_{560} (106, 174).

Methanofuran, the Coenzyme of Formylation

Although progress had been made in other areas of C_1 reduction, nothing was known in 1980 about the activation and reduction of CO_2 to the formyl level. James Romesser observed that cell extracts could be resolved for a factor that was required for the reduction of carbon dioxide to methane after passage through a short Sephadex G-25 column. This factor was partially purified and named the carbon dioxide reduction factor, CDR (113). John Leigh took up the study of this factor for his thesis project, a study that would take five years. He resolved the fraction from methanopterin and showed that the CDR factor had properties that were quite distinct (91), including the lack of absorption in the visible or long UV spectrum. This finding meant that each fraction from column chromatography had to be tested in a methanogenic assay to locate the active fraction, a laborious and time consuming process. After analysis of its hydrolytic fragments, a structure for the coenzyme was proposed (89) as 4[N-(4,5,7-tricarboxyheptanoyl-γ-L-glutamyl-γ-L-glutamyl)-ρ-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan.

The counsel of K. L. Rinehart, Jr. was essential to the success of the project. The compound was essentially a long linear molecule with a hydrophilic tetracarboxylic acid moiety on one end and a furan ring with a primary amine on the other end. Aharon Oren suggested the name methanofuran, and we liked it. Later, the methanofuran of *Methanosarcina barkeri* was found to contain glutamyl units instead of the tetracarboxylic acid structure (12). But how did the compound function? John Leigh thought it might be involved in electron transport. But now and then I would ask, "John, ol' boy, does CDR carry a C_1 group?" It was great fun when he showed that formyl-methanofuran was the product of CO_2 activation and reduction (90) and that the formyl group was carried on the primary amine of the furan moiety. A new method of CO_2 fixation had been discovered.

7-Mercaptoheptanoylthreonine Phosphate, the Coenzyme That Donates Electrons to the Methylreductase

Robert Gunsalus & S. Tandon developed a successful procedure for anaerobic column chromatography of cell extract at room temperature (60). Gunsalus separated the methylreductase system into three fractions, each of which was required for reconstruction of the methylreductase reaction from CH_3-S-CoM and molecular hydrogen (64). Component A consisted of large oxygen-sensitive, heat-labile protein complexes with hydrogenase activity. Component B was an oxygen-labile, heat resistant, dialyzable cofactor. Component C was an oxygen-stable, heat-labile protein. Over 10 years passed between the discovery of component B and elucidation of its structure, the most frustrating experience of my academic life. Component B, the small, heat-stable molecule that was absolutely required for reconstitution of the methylreductase reaction was rather stable in crude preparations. However, the factor proved to be highly unstable on fractionation. Ralph Tanner invested several years in attempts to purify the factor to homogeneity but finally had to settle for a partially characterized compound. Kenneth Noll then continued studies on component B with four years of discouraging results. As with methanofuran, no spectral properties were available that could be used to follow the compound during fractionation. The only assay available was methanogenesis by the reconstituted methylreductase system. Years of frustration centered around loss of activity during fractionation. A complex molecule appeared to be decomposing during fractionation, but no conditions could be found to prevent the decomposition. One day, Kenneth Noll showed me a recording from HPLC that exhibited an isolated peak about 2 mm high, and he said with an air of resignation in his voice, "That's B." This was what I had been waiting for. We increased the manpower and ran the column repeatedly until we had enough material to make some measurements.

The breakthrough had been made. We would eventually find that the compound was not decomposing; instead it was forming heterodisulfides during fractionation with any HS-compound that was available. Elucidation of the structure by Ken Noll as 7-mercaptoheptanoylthreonine phosphate, HS-HTP (103), with the counsel of K. L. Rinehart and its confirmation through chemical synthesis by Noll & Mark Donnelly (102) were rewarding experiences after 10 years of frustration. Tanner studied the growth factor required by *Methanomicrobium mobile* (124), and my last graduate student, Carla Kuhner, extended these studies to show that the unknown growth factor is HS-HTP.

Enzymology of Methanogenesis from CO_2 and H_2

Weaving the role of these six new coenzymes into a metabolic pathway for the reduction of carbon dioxide to methane was an intriguing experience that

spanned about 15 years. Some of the coenzymes were C_1 carriers; others were electron carriers. It became evident that nature had chosen the strategy of keeping the C_1 group bound to a coenzyme as it was sequentially reduced. The first reaction of the methanogenic pathway to be studied was the CH_3-S-CoM methylreductase reaction (62, 64, 94, 127). Component C was shown to be a protein of M_r 300,000 with an $\alpha_2\beta_2\gamma_2$ subunit configuration with 2 mol of F_{430} (38, 39). The enzyme contained CoM, and later Patricia Hartzell determined by the *M. brevibacter* assay that a preparation of component C prepared in Walsh's laboratory contained 2 mol of bound CoM per mol of protein (71). Hartzell et al (66) could find no stoichiometry for CoM incorporation into component C. Kenneth Noll showed that component C also contained bound component B (HS-HTP) (104). Although we could find no evidence for cobamides or corrins in component C, Whitman (155) found that the addition of corrins to the reaction mixture activated the methylreductase reaction. He studied the activation of the methylreductase by ATP (153) as well as its inhibition by corrins (154). Hartzell performed a comparative study of component C from various methanogens and revealed minor differences (68). Later, Pierre Rouvière used the variation in the subunits of component C to construct a taxonomy for the methanogens (118) that is in remarkable agreement with that derived from analysis of 16S rRNA.

Because component C from *Methanobacterium thermoautotrophicum* strain ΔH required component A proteins for conversion to the active state, Hartzell was able to dissociate and purify separately the α, β, and γ subunits as well as F_{430}. She then showed that these constitutents could be reassembled into component C, which in the presence the A proteins showed a 70% recovery of the methylreductase activity (69). F_{430} was absolutely required, showing for the first time that it really was a coenzyme in the methylreductase reaction. Homogeneous component C from strain ΔH required protein fraction component A and coenzyme component B for activity (64). In a series of very demanding experiments, David Nagle (101) separated component A into three protein fractions, A1, A2, and A3. This was an especially difficult task because the only assay available was the reconstituted methylreductase system. Each fraction that was purified required three other active fractions for its assay. Fraction A3 was found to be specifically inhibited by 2',3'dialdehyde of ATP (119). Later, fraction A3 was separated into two protein fractions, and A2 was purified to homogeneity by Rouvière (117, 121). So far, no specific enzymatic reaction has been assigned to these fractions, but A2 and A3 appear to be involved in converting inactive component C (C_i) to its active form (C_a) (120). Attempts to simplify the assay (67) or study the reductive activation (116) did not produce a breakthrough. A breakthrough had occurred when Ankel-Fuchs in Thauer's laboratory prepared an active form of component C from the Marburg strain. These ex-

periments proved what everyone had suspected: that component C was the methylreductase (2). We sent Ankel-Fuchs a sample of HS-HTP to see if it substituted for component B in a reaction mixture with C_a; it did (1). Because HS-HTP worked in this ATP-free system with C_a, it obviously was not adenylated. When 7-[methylthio]heptanoylthreonine phosphate was tested, the methyl group was not reduced to methane (105). Noll (105) showed that HS-HTP was the electron donor for CH_3-S-CoM reduction, and Bobik & Olson (13) found that the products of the reaction were methane and the heterodisuflide, CoM-S-S-HTP. Similar results were found in Thauer's laboratory (40, 41). Finally, after nearly 20 years of work on CoM, we could write a reaction for the formation of methane:

$$CH_3\text{-S-CoM} + \text{HS-HTP} \rightarrow CH_4 + \text{CoM-S-S-HTP}.$$

In 1977, Robert Gunsalus made a curious discovery; the conversion of carbon dioxide to methane was greatly stimulated by the addition of CH_3-S-CoM to the reaction mixture (61). We believed this observation meant that in some manner the terminal methylreductase reaction was coupled to the activation of carbon dioxide and named this phenomenon the RPG effect after his initials. Romesser showed that the RPG effect in three species of methanogens could be initiated by methanogenic precursors (114). With the discovery that CoM-S-S-HTP was a product of the methylreductase reaction, Bobik found that this heterodisulfide specifically initiated the RPG effect in the absence of the methylreductase reaction (14). At present, the mechanisms of the activation of CO_2 reduction by the heterodisulfide is not understood, but Bobik has proposed that CoM-S-S-HTP activates an electron carrier that donates electrons to formylmethanofuran dehydrogenase (15). Another curious observation, made by Romesser (111), was that addition of certain intermediates of the citric acid cycle such as malate initiated the RPG effect. At that time, we believed that certain carbon atoms of these compounds, because of metabolic reactions, eventually ended up as a methyl group on CoM. However, Bobik discovered a very unusual fumarate reductase, a thiol-driven reaction in extracts of strain ΔH that required both HS-CoM and HS-HTP as electron donors in which succinate and CoM-S-S-HTP are formed as products (16). Thus, CoM-S-S-HTP may be formed by more than one reaction. Bobik also showed that the activation of CO_2 by the heterodisulfide resulted in the production of formylmethanofuran (15).

When Mark Donnelly joined the laboratory, he was attracted to the fate of the formyl group on formylmethanofuran. Jorge Escalante had just shown that tetrahydromethanopterin (H_4MPT) reacted with formaldehyde to form methylene-H_4MPT, which could be oxidized by extracts to form methenyl-H_4MPT (45). Transfer of the formyl group from formylmethanofuran to H_4MPT

to produce formyl-H_4MPT seemed to be a logical reaction to look for, and Donnelly found it. He developed an assay to follow the formyltransferase reaction and purified the enzyme to homogeneity (34). Formation of 5-formyl-pterin was a novel enzymatic reaction, one not found in folate biochemistry. Subsequently, the formyltransferase was cloned and sequenced by Anthony DiMarco with Karen Sment from Jordan Konisky's laboratory (32). The enzyme was expressed in *E. coli* and was found to be active at 60°C. A search of gene banks revealed that the enzyme is unique and unrelated to any previously described enzyme. Donnelly & Escalante (33) found the 5-10 methenyl-H_4MPT cyclohydrolase and showed that 5-formyl-H_4MPT was formed as a product; DiMarco & Donnelly (31) purified the enzyme to homogeneity and described its properties. By use of both homogeneous enzymes, Donnelly then showed that formylmethanofuran could be converted to methenyl-H_4MPT (34), indicating that these two enzymes were components of the methanogenic pathway.

Escalante (45) had shown that methylene-H_4MPT could be oxidized to methenyl-H_4MPT, and Hartzell et al (70) found a methylene-H_4MPT dehydrogenase that required coenzyme F_{420} to carry out the oxidoreductase reaction. Aharon Oren & Jeffrey Hoyt (75) purified and described the H_4MPT-serine transhydroxymethylase that formed methylene-H_4MPT. Escalante (47) had shown that the methylene group of methylene-H_4MPT could be reduced chemically to form 5-methyl-H_4MPT, but had difficulty showing the enzymatically catalyzed reduction; however, the methyl group of 5-methyl H_4MPT was converted to methane by cell extracts (45, 47). Current concepts are that the methyltransferase contains a cobamide group in which the cobalt atom accepts the methyl group from methyl-H_4MPT (108); this enzyme was named methyltransferase-1. Earlier, Taylor reported the purification of an enzyme that transferred the methyl group from methylcobalamin to HS-CoM (127). Taylor's enzyme is now believed to transfer the methyl group from the cobamide of methyltransferase-1 to HS-CoM. Evidence for the methylene-H_4MPT reductase and its requirement for reduced F_{420} was obtained by TeBrömmelstroet et al (129) in Vogel's laboratory. Shapiro (122) showed that CH_3-S-CoM was an intermediate in methanogenesis from methanol by *Methanosarcina*, and Baresi (9) documented levels of coenzyme F_{420}, CoM, hydrogenase, and CH_3-S-CoM reductase in acetate-grown cells. So, it appears that the pathway we had proposed (30, 79, 120) for methanogenesis from carbon dioxide and hydrogen is essentially correct.

Microbial Diversity

Gallionella had interested me as a graduate student, so in 1954, I looked forward to studying this iron bacterium in Pacific Grove. van Niel was enthusiastic, and upon my arrival handed me a file of reprints, saying as he

pointed to one, "This is an important paper. Unfortunately it is in Russian, but it has a good German summary." He loved to remind American students of their language limitations. *Gallionella* secretes a twisted ribbon-like sessile stalk of ferric hydroxide, and van Niel had suggestions about setting up enrichments in Carrell flasks. I followed his suggestions, and, in a few weeks, had my first successful enrichments; van Niel seemed impressed. These cultures did not survive the return trip to Illinois, so I was anxious to find a source of organisms.

During a weekend at Turkey Run State Park in Indiana, Gret saved the day by finding rusty patches along the Rocky Hollow trail; these proved to be an excellent source of *Gallionella*. Al Vatter took some electron micrographs that showed that the stalks were not solid ribbons but were composed of many strands secreted from one side of the cell (149). We developed a defined medium, and Sonia Kucera worked on *Gallionella* for her MS thesis (86). I prepared an article on iron bacteria for the *Journal of the American Water Works Association* (164); over the years I would take some kidding from scientific colleagues for publishing in this "prestigious" journal. I used this article to encourage water plant operators to send me samples of problem organisms. A beautiful example of the chlamydobacterium, *Crenothrix*, arrived from Sweden (165), but I could not cultivate it. Another sample contained a large *Leptothrix*. Because of problems with obtaining pure cultures and with mass culture, these organisms had to be abandoned as serious research subjects, but iron bacteria continue to be a scientific hobby. Our children continue to send me samples of rusty deposits they find in nature.

Phototrophs, my favorite enrichments in the organism course, were always fun to isolate, and, when Al Vatter became interested in the ultrastructure of *Rhodospirillum rubrum*, I suggested that we collaborate on a study of representative photosynthetic bacteria to discern their cellular anatomy. We published a pioneering article on the structure of photosynthetic bacteria, showing that *R. rubrum*, *Rhodopseudomonas sphaeroides*, and *Chromatium* all contained differentiated membranes, chromatophores (150). We also showed that *Chlorobium* was different, but we missed the chlorosome. While on a summer fellowship at the University of Washington, I set up enrichments for *Rhodomicrobium vannielii*, a most unusual phototroph discovered by Howard Douglas. We were surprised to find that the organism was motile (35) and that it contained lamellae rather than chromatophores (148). When Robert Uffen arrived for postdoctoral study, I suggested that he set up enrichments for methanogens that could grow on carbon monoxide. A technique was developed for streaking enrichment cultures on agar in bottle plates to which we could add an atmosphere of carbon monoxoide. Much later an improved bottle plate was developed and Bellco Glass produced it (74). Uffen could detect no methane in the bottle plates, but one plate contained a large red

colony. I knew that Howard Gest and other investigators had been trying to grow phototrophs anaerobically in the dark without success. The organism proved to be *Rhodopseudomonas palustris*, a purple nonsulfur phototroph. A very careful study to insure that utilizable radiation was not available to the cells revealed that by use of pyruvate and H_2, representative nonsulfur phototrophs also could be cultured anaerobically in the dark indefinitely and that their photosynthetic apparatus could not be differentiated from that of anaerobic light-grown cells (134). Mutants also were studied (134a).

Beggiatoa, with its gliding trichomes stuffed with sulfur, was a fascination for me. I thought it would be fun to isolate, mass culture, and study the metabolism of this organism. We followed the method of Cataldi for isolation by allowing a trichome to glide over an agar surface; a cut-out agar block with the trichome on it was then transferred to sterile medium. Lois Faust developed a defined medium for the organism. By 1959, I realized that it was unrealistic to ever expect to mass culture this organism in quantities required for enzyme studies, so I documented its characteristics with photographs and motion pictures (50) and used these in teaching for many years.

Sarcina ventriculi, which carries out an alcoholic fermention of sugar at pH 2.5, was studied by Ercolé Canale-Parola, my second PhD student. The organism was isolated from sediments of Boneyard Creek, a somewhat polluted stream that runs through our campus. The real challenge was to maintain the organism, for it could die quickly if the culture was not transferred properly. He developed a stock culture technique (24), evolved a synthetic medium (25), and studied the localization of cellulose (23) as well as its synthesis (26).

Streptococcus allantoicus was isolated by Ray Valentine from a duck pond at Monticello, Ill., to study allantoin degradation. H. A. Barker had discovered the organism and suggested that possibly one of the ureido groups of allantoin could be cleaved in an energy-yielding reaction. Valentine discovered the enzyme, oxamic transcarbamylase (141), and showed that the phosphorolysis of oxalurate yielded oxamate and carbamyl phosphate, the latter compound donating a phosphate group to ADP in a kinase reaction to yield ATP (142). In studying the phosphate-dependent degradation of urea, ureidoglycolate was found to be an intermediate (143, 144), and its NAD-dependent oxidation to oxalurate was proposed as the only oxidative step carried out by the organism when it grew anaerobically on allantoin (135). Elizabeth Gaudy (55, 58) characterized the enzyme ureidoglycolate synthetase for her thesis and implicated the enzyme in the allantoin degradation pathway. Robert Bojanowski & Gaudy (17) characterized the oxamic transcarbamylase, and Valentine & Harvey Drucker (138) documented the conversion of glyoxylate to tartronic semialdehyde and CO_2.

Sphaerotilus natans was studied by Elizabeth Gaudy for her MS thesis. In

rich media, sheath formation by this chlamydobacterium ceased and the organism produced copious amounts of slime (56). The slime was purified and found to be composed of equal amounts of fucose, galactose, glucose, and glucuronic acid; we suggested that the latter two components occurred as an aldobiuronic acid (57). We then received a letter from Michael Heidelberger, who suggested that the aldobiuronic acid could be identified using antibodies that he had. The aldobiuronic acid precipitated with Type III and Type VII antipneumococcal horse sera and was identified as cellobiuronic acid (73).

Arthrobacter crystallopoietes was brought to my laboratory by a postdoctoral student, Jerald Ensign. *Arthrobacter* goes through a rod to coccus differentiation during growth, and he wished to study this phenomenon. He developed a chemically defined medium in which the organism grew only in the coccoid form. He discovered that addition of certain amino acids or organic acids to the defined medium resulted in the formation of the rod-shaped stage (42).

Myxobacter strain AL-1 was isolated by Ensign (43) by placing soil inocula on a lawn of *Arthrobacter*, where the myxobacter produced large clear areas; the lytic enzyme was found to readily lyse a number of gram-positive species (43). The enzyme was found to be a small protease that hydrolyzed proteins as well as peptide bonds in the glycosaminopeptide of cell walls (44). Richard Jackson studied the amino acid composition and characterized the enzyme (78). Strominger found the enzyme, Myxobacter AL-1 Protease I, to be valuable in determining the structure of the cell walls of gram-positive bacteria. A second protease, Myxobacter AL-1 Protease II, was discovered when the enzyme crystallized in certain fractions as it eluted from a column. We were somewhat stunned; usually an enzyme yields to crystallization only after a battle, but here we had crystals and no assay. Marilyn Wingard (158) determined that the enzyme was a protease that had the unusual specificity to cleave the peptide bond on the amino side of lysine. A third enzyme was discovered when in the course of dialyzing myxobacter extracts the dialysis tubing disintegrated. Allan Hedges (72) showed that this enzyme had both β-1,4 gluconase as well as chitosanase activity.

Clostridium tetanomorphum was studied by Robert Twarog, who showed that the organism makes only 1 mol of ATP per mol of glutamate and that ATP is generted via phosphostransbutyrylase and butyrate kinase reactions (130, 131).

Geodermatophilus was brought to the laboratory by Edward Ishiguro; he had found the organism in soil obtained from the lower reaches of Mt. Everest. From a clump of large coccoid cells, certain cells differentiated small motile rods, which multiplied as rods but later differentiated to cocci, forming large clumps. He studied this phenomenon and found that the life cycle could

be controlled; a factor in certain batches of Tryptose (Difco) was required for differentiation to the motile-rod form (76). This factor was difficult to purify because the growth-differentiation assay was not easy to quantify. After a few years, the factor was in hand and the assay was reproducible (77), but Ishiguro was rather depressed; the factor was an inorganic cation (NH_4^+) and other inorganic as well as organic cations induced morphogenesis.

Methanobacterium thermoautotrophicum was isolated by Gregory Zeikus. In 1970, Leon Campbell encouraged me to set up some enrichments for thermophilic methanogens. These enrichments readily produce methane, but I could not pursue a successful isolation at the time. When Zeikus came to the laboratory, I suggested that he continue this project of isolating a thermophilic methanogen. He proceeded to enrich and isolate an organism from sewage sludge that grew well at 60–65°C. In spite of our poor latin scholarship (181), isolation of this organism, *Methanobacterium thermoautotrophicum* strain ΔH, was a major breakthrough. The organism had an interesting ultrastructure (182). Strain ΔH became the backbone of my research program. It could be readily scaled up to the 200-liter fermentor stage, it grew more rapidly than mesophilic strains, and, above all, enzymes were stable at room temperatures, 40° below the optimal growth temperature.

Methanogenium cariacii and *Methanogenium marisnigri* were isolated by James Romesser (115). Since I was a child, I have had an unfortunate propensity for motion sickness, so when Holger Jannasch invited me to join an expedition to the Cariaco Trench and later to the Black Sea, I was forced to decline. However, Romesser was pleased at the possibility for adventure and returned with samples from water columns of these anaerobic habitats.

Methanococcus voltae was sent to us by J. M. Ward, who had isolated it for his MS thesis, and we decided to name it after Alessandro Volta, who first described the formation of "combustible air" in sediments (3). Whitman and colleagues defined its nutrition and carbon metabolism (151) as well as its plating efficiency (82). We suggested that this organism might be the organism of choice for initiating genetic studies with methanogens.

Methanococcus jannaschii was isolated by John Leigh from samples obtained by Holger Jannasch at a geothermal vent on the east Pacific rise by use of the submersible, Alvin. This unusual organism grew optimally at 85°C with a generation time of 25 min. Its properties were reported by Jones et al (81).

Methanogenium thermophilum was isolated by Friedrich Widdel. He had stunned the field by showing that methanogens could oxidize alcohols and reduce CO_2 to CH_4. In Urbana, he isolated mesophilic and thermophilic alcohol-oxidizing methanogens and with Rouvière studied their taxonomy (156). He showed that F_{420} served as an electron acceptor for alcohol dehydrognease (157).

Acetobacterium woodii was discovered in enrichments set up for methanogens in which a gas mixture of 80% H_2:20% CO_2 was bubbled through a series of tubes that had been inoculated with sediment from Crystal Lake, Urbana, Ill. To save gas, the effluent of one tube was connected to the inlet of the next tube. The tubes were placed in a fume hood. About a month later, I happened to notice these tubes with their slowly bubbling contents; I had forgotten them. I could find no evidence of methane formation, but the black rubber stoppers were swollen and gnarled, and the amount of acetic acid coming out of the tubes was fantastic. This was obviously not the way to enrich for methanogens! At that time, no H_2-oxidizing, CO_2-reducing acetogen was in culture. One summer at Woods Hole, we set up enrichments from sediments at Oyster Pond Inlet, and Balch succeeded in isolating a cocco-bacillus-like organism. We named the organism after Harland Wood (5), who had spent 20 years trying to figure out how acetic acid was synthesized from CO_2, and presented him with a picture of the organism on his 70th birthday. Siegfried Schoberth thoroughly documented the substrate range and properties of the organism, and Ralph Tanner determined the presence of tetradhydrofolate enzymes in the organism (125). *A. woodii* opened the modern era of hydrogen-oxiding acetogens, the ecological importance of which is becoming increasingly appreciated. Later, John Leigh isolated a thermophilic acetogen on H_2 and CO_2 from sediments of Lake Kivu, Africa. We named this organism *Acetogenium kivui* (88).

Methanospirillum hungatei, strain JF, was isolated by Gregory Ferry from a stable consortium of cells that degraded benzoate anaerobically to CH_4 and CO_2 (52). He resolved the culture into three organisms. One was a pseudomonad that used benzoate as its substrate. Another organism was a spiral-shaped methanogen that grew on H_2 and CO_2 or formate. The other organism could not be isolated but was an acetophilic methanogen. The spirillum was similar to one described by Paul Smith in an abstract, but a publication never appeared, so we collaborated on its description and named the organism *Methanospirillum hungatei*, in honor of Robert Hungate (51). Later, the nutritional and biochemical characteristics of *M. hungatei* were described (53).

Josef Winter joined the laboratory and achieved complete degradation of carbohydrate to methane by a syntrophic culture of *A. woodii* and *M. barkeri* (160). In another study, he followed methane formation from fructose by *A. woodii* and other methanogens in a chemostat (159). I believe he was the first person to use a chemostat to study methanogens. He returned to Otto Kandler's laboratory and trained his colleagues to use the techniques developed by Balch. One brilliant pupil was Karl Stetter. Other consortia were studied by Jack Jones and Jean Pierre Guyot (80).

While on sabbatical leave in 1975, Norbert Pfenning suggested that I

should isolate and study a spirillum-like organism that was persistent in enrichment cultures of *Desulfuromonas*. We named the organism spirillum 5175 and found that it could grow anaerobically by the oxidation of formate with the reduction of fumarate to succinate. It could also use H_2S as electron donor for this reaction. In addition, it could anaerobically oxidize hydrogen or formate and reduce elemental sulfur, thiosulfate, or sulfite, but not sulfate. Since *Chlorobium* does not use formate, we set up a syntrophic culture in which the spirillum oxidized formate and reduced elemental sulfur to H_2S; *Chlorobium* used the H_2S as its electron donor, producing elemental sulfur. Sulfur was limiting in the medium and was recycled through H_2S and elemental sulfur, so neither organism could grow alone, but together they grew beautifully (168).

Richard Blakemore discovered magnetotactic bacteria in sediments that had been collected at Woods Hole, but he was unable to pursue their isolation. Before his arrival in Urbana as a postdoctoral student, I spent the summer studying the survival of these organisms from enrichments in various media and conditions. Their survival was a matter of minutes or hours not days. When he arrived in September, 1976, I suggested that we set Christmas as a goal for achieving the first pure culture; by February he had a magnetic spirillum in hand (10). It would be 10 years before a second species would be captured by others. While on a von Humboldt Fellowship in Germany, I continued the study of magnetic bacteria; why were they so difficult to isolate? An undergraduate student in Rolf Thauer's laboratory, Alfred Spormann, was interested, and we found an excellent natural enrichment. Using reducing agents, we showed that chemotaxis could maintain a magnetically oriented cell at a certain oxygen tension, explaining why certain magnetotactic cells may not follow the magnetic lines of force deep into anaerobic sediments (123). I was able to perfect a capillary racetrack method for separating magnetic bacteria from typical bacteria (169), but an appropriate medium for their culture could not be formulated.

Reflections

Some believe it takes as much work to study an unimportant problem as an important one, so one should only work on important problems. Perceived important problems are usually in good hands, which means that most of us, fortunately, have the freedom to work on what interests us, and sometimes this turns out to be important. My goal has been to get as many persons as possible interested in working on methanogens. After a sabbatical leave in Urbana, Godfried Vogels returned to start his own program on methanogens at Nijmegen. Rolf Thauer became interested in methanogens after a two-week visit to Urbana. Josef Winter returned to Kandler's laboratory in Munich. Ziegfried Schoberth returned to Gottschalk's laboratory at Göttingen. Many

individuals were trained by Balch, including visitors from Ottawa. Chris Walsh, Orme-Johnson, and students visited from MIT. John Reeve and students visited from Columbus. Jordan Konisky was encouraged to start his own program at Urbana. By 1982, there was a critical mass of investigators, and we began to organize our first Gordon Conference on methanogenesis.

During the years of teaching the organisms course at Urbana, I was frequently reminded of the limitations of formal laboratory scheduling where a two- to three-hour laboratory is wedged between classes, and where students have limited opportunities to make discoveries. I had always wanted to try a van Niel–type summer course, so when the opportunity came to be Director of the Microbiology Course at the Marine Biological Laboratory at Woods Hole, I asked Peter Greenberg, who would bring genetic and molecular components to the course, to join me as codirector with the purpose of starting a 1985 version of a van Niel–type summer organisms course. He agreed. We asked Bernhard Schink from Konstanz to join us for the first summer, and Carrie Harwood was course coordinator. Holger Jannasch was instrumental in urging students on the national and international scene to take the course. In subsequent years, we were joined by Norbert Pfenning, Friedrich Widdel, Bernhard, and Andrew Kropinski as course coordinator. We found that the Volta experiment on the third evening of the course not only focused interest on anaerobic physiology but was a great social mixer for students and staff. Student response and faculty dedication made these five summers the most satisfying teaching experiences of my academic career.

Over the years, I have formulated a few truisms that I refer to as Wolfe's Laws of Thermodynamics: 1st Law: Unpublished data do not improve with age. 2nd Law: If you are first on the scene, it is easy to make discoveries. 3rd Law: The emotion generated in scientific discussion increases proportionally with the softness of the data being discussed. 4th Law: If you join a parade, you become one of the marchers. Graduate students like to join highly visible parades, to be part of the current scene, not realizing that their visibility is reduced by the number of marchers. As an independent investigator, each should realize the importance of choosing a problem that isn't moving and move it.

Throughout my academic life, I have more or less been driven in some manner "to go back to the lab." This common syndrome of persons in science needing to be in the lab could be simply a security blanket or a necessary component of scientific survival. I have felt that, for me, the latter was the case, coupled with a feeling that possibly my presence somehow might encourage students. Gret has spent many lonely evenings sharing her life with the lab, and her support was pivotal. Our children, Danny, Jon, and Sue, shared a portion of their lives with the lab. However, one of the enjoyable aspects of academic life is the sabbatical; we have had interesting sabbaticals

in England, Hawaii, France, and Germany. My last sabbatical, a short one, was spent with John Breznak, where I was introduced to the fascinating world of anaerobic protozoa from termites.

As these scientific memoirs have shown, I was fortunate to have a series of talented graduate and postdoctoral students to work with me. I thank them, my colleagues, Gret, and our children for making this experience a rewarding one. In operating my laboratory, my purpose has not been to play the role of the brilliant intellectual leader, but rather to stay in the background and try to create an atmosphere in which students could develop into independent investigators; this, for me, is what it's all about.

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