

Life without air

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An early exposure to lipid biochemistry in the laboratory of Konrad Bloch resulted in a fascination with the biosynthesis, structures, and functions of bacterial lipids. The discovery of plasmalogens (1-alk-1'-enyl, 2-acyl phospholipids) in anaerobic Gram-positive bacteria led to studies on the physical chemistry of these lipids and the cellular regulation of membrane lipid polymorphism in bacteria. Later studies in several laboratories showed that the formation of the alk-1-enyl ether bond involves an aerobic process in animal cells and thus is fundamentally different from that in anaerobic organisms. Our work provides evidence for an anaerobic process in which plasmalogens are formed from their corresponding diacyl lipids. Studies on the roles of phospholipases in *Listeria monocytogenes* revealed distinctions between its phospholipases and those previously discovered in other bacteria and showed how the *Listeria* enzymes are uniquely fitted to the intracellular lifestyle of this significant human pathogen.

Anerobiosis, life without air, was the focus of my work during my first postdoctoral position at the National Institutes of Health (NIH) with the legendary Earl R. Stadtman. That experience led to a second postdoctoral fellowship in the laboratory of Konrad Bloch at Harvard, where one of the goals was to understand how anaerobes make unsaturated fatty acids. The lipids of anaerobes have remained an object of fascination for many years.

But I am getting ahead of myself. First some background of how I got from Brooklyn, New York, to those eminent institutions. My parents were Samuel and Ida Cohen Goldfine. Sam had immigrated to this country from Vilnius, Lithuania, around 1900, joining three brothers and a sister. My dad was not forthcoming about his early life and, regretfully, I did not inquire. By the time I was born, he had owned and lost a millinery business in the Great Depression, had three children, and lost his first wife to cancer. He then married my mother, who was 10 years younger. My mother had also come to America from Eastern Europe. Her father was a potter in the city of Augustow in northeastern Poland. Her two sisters and two brothers had also emigrated near the turn of the last century.

After moves from Brooklyn to Manhattan and then back, my family settled in the Brownsville section of Brooklyn to be near

one of my aunts. At that time, Brownsville was a predominantly Jewish neighborhood, about 30 minutes by subway from midtown Manhattan. After completing elementary school and junior high, I was ready to enter 10th grade and took the test to go to Stuyvesant High School, then, as now, one of the preeminent schools in the city, dedicated to science education. I was accepted and rode the subway for about 4 days before deciding I would rather go to my local high school, Thomas Jefferson. I cannot recall the feeling I had at age 14 that Stuyvesant was not a good fit for me; despite the disapproval of an assistant to the principal at Jefferson, I made the move and have never regretted it. When I eventually matriculated at the City College of New York (CCNY), I found myself in classes with former classmates who had graduated from Stuyvesant and never felt that my education at Jefferson was lacking. At that time, New York City high schools had many excellent teachers who joined the profession during the Great Depression for economic reasons. During my teenage years, I, like many microbiologists of my generation, read Paul de Kruif's book *Microbe Hunters* and was inspired by his romantic descriptions of the lives of eminent microbiologists, including Louis Pasteur, Robert Koch, Walter Reed, and David Bruce. In my high school yearbook, I noted my ambition to become a bacteriologist.

City College had students from all five boroughs and many ethnicities. It had strong departments in the sciences, and I enrolled as a biology major. Fortunately, I had the opportunity to take many courses in chemistry as well. Although traveling to City College required a long ride on the subway, I usually had a seat and spent the time studying and reading books. City College had been a hotbed of leftist thinking in the turbulent 1930s, but the postwar influx of WWII veterans resulted in a much less strident liberalism, which suited my temperament.

As I entered my senior year in 1952, I was drawn to biochemistry by three members of the chemistry department: Benjamin Harrow, chair of the department, Ernest Borek, and most importantly Abraham (Abe) Mazur. Abe was responsible for stimulating a large number of City students to pursue graduate studies in biochemistry. Among these were Ted Breitman, Nathan Brot, Alan Peterkofsky, Jack Preiss, and the two Weissbach brothers, Arthur and Herbert (Fig. 1). He believed correctly that biochemistry would play an increasingly important role in the development of the biological sciences. He parceled his promising students out to biochemistry departments that contained former students or colleagues, and he advised me to apply to the University of Chicago, where several people he knew were among the biochemistry faculty. I also applied to and was accepted at several other strong institutions, but with Abe's guidance I chose Chicago.

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"La fermentation . . . C'est la vie sans air, c'est la vie sans oxygène libre."—Louis Pasteur

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Figure 1. City College of New York alumni at a conference to honor Earl Stadtman at the National Institutes of Health in 2009. Left to right, Alan Peterkofsky, the author, Herbert Weissbach, and Arthur Weissbach. Elliott Schiffman, not a CCNY alumnus, was a colleague of Earl Stadtman at NIH but is now retired.

Chicago

Aside from summer jobs in the Catskills (the so-called Borscht Belt) and in the Adirondacks, I had never been away from home without a parent, but to me Chicago was just another big city. Biochemistry at the university was housed in the well-worn Abbott Hall, and several courses like biochemistry and physiology were taken with first-year medical students. The department was led by Earl A. Evans, Jr., who had shown that animal cells could fix CO_2 to form carbohydrates. He appointed a number of outstanding biochemists, including Albert Lehninger, Eugene Kennedy, and future Nobel Laureate Konrad Bloch. Within the department, he had formed a bacteriophage group under the sponsorship of the March of Dimes, which included Lloyd Kozloff and Frank Putnam.

The University of Chicago had been the site of the first sustained nuclear chain reaction, led by Enrico Fermi, which led to strong emphasis on the use of radioisotopes to study biochemical reactions and pathways. During my second year, I looked around for potential thesis advisors and had an interview with Eugene Kennedy, who was just establishing his reputation with the discovery that CTP played an intrinsic role in phospholipid biosynthesis in animal tissues through the formation of CDP-choline. Gene said he did not have space for a graduate student, and I suspect he did not think I would be a good fit. I next applied to Evans, who told me that he wanted to branch out from phage work to do research on the polio virus, since the March of Dimes—formed under the aegis of President Franklin D. Roosevelt—was dedicated to finding a cure or at least a way to prevent polio.

We discussed setting up a new laboratory in the Fermi Institute in space recently vacated by Harold Urey. I would have a

small laboratory with a tissue culture room and a full-time technician, and I would share an office with Ray Koppelman, who had completed his Ph.D. in biochemistry in the phage group. Ray was no longer interested in doing research and was dedicating himself to teaching undergraduates. He would keep an eye on me and, I assume, report back to Evans. Before starting work with live polio virus, I learned how to grow HeLa cells, which had only recently been shown to be useful for studies on animal viruses. The cells were obtained from another university laboratory; no commercial sources were available in 1955. We started by making our own Eagle's medium using calf serum that I obtained at a local slaughterhouse. My initial studies were on the metabolism of cells grown in culture, and I, not surprisingly, found that HeLa cells, like other cancer cells, carried out robust glycolysis and produced large amounts of lactic acid. Having gained confidence that I could continue the cultivation of HeLa cells, I began the study of nucleic acid synthesis in cells infected with polio virus. These studies resulted in a thin (by present-day standards) thesis and a paper in the *Journal of Biological Chemistry* (1). Briefly, after infection of HeLa cells with polio virus, RNA synthesis continued, but DNA synthesis shut down.

Postdoctorals, the Stadtman and Bloch laboratories

Months before completing my thesis, I began to consider where I would go for postdoctoral studies. I considered working with, among others, H. A. Barker at Berkeley, Arthur Kornberg (then) at Washington University, and Earl Stadtman at the NIH. I met Earl at a Federation of American Societies for Experimental Biology Meeting in Chicago and inquired about the possibility of joining his laboratory. He said he could accommo-

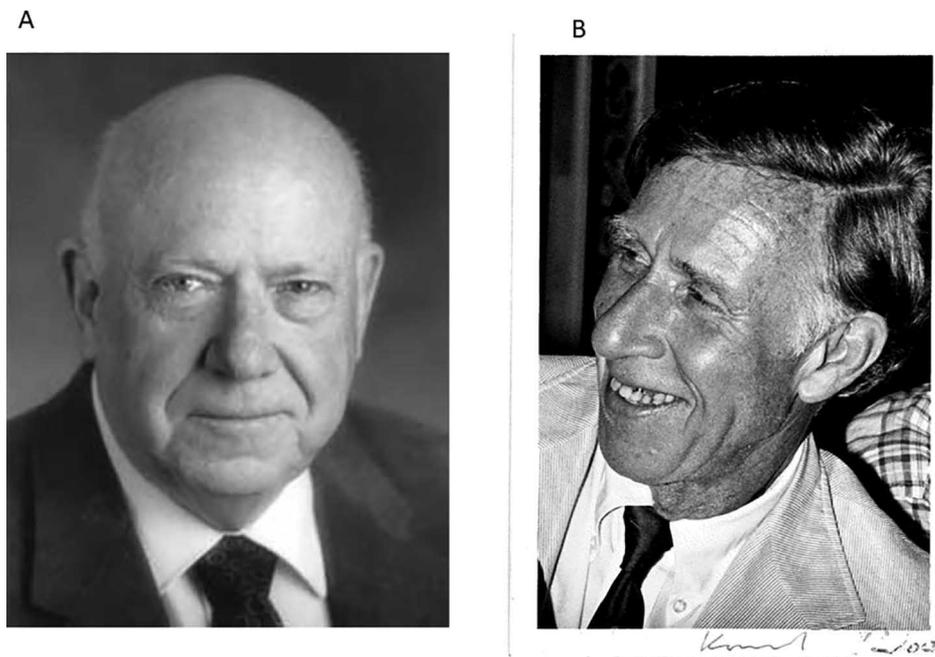


Figure 2. A, Earl Stadtman (Office of NIH History). B, Konrad Bloch.

date me, but I would have to obtain my own funding. I briefly considered a commission in the Public Health Service but was told that, if I accepted it, I would be assigned to another laboratory. So in January 1958, I moved to the NIH campus in Bethesda, Maryland, and joined the Stadtman lab in the basement of building 3, having obtained a postdoctoral fellowship from the NIH.

Earl and his wife, Thressa (Terry), had separate research programs, but their common interest in the physiology of anaerobic bacteria resulted in joint laboratory meetings and shared social interactions (Fig. 2). They had no children, and their lives revolved around their research. Earl was very soft-spoken and—although I did not realize it at the time—I was beginning to lose hearing in one ear, which made communication difficult. We settled on studies of the conversion of β -alanine to propionic acid by *Clostridium propionicum*. Roy Vagelos had been working on propionic acid formation in the laboratory, and Earl suggested I start by learning how to do a tetrazolium dye reduction assay from Roy, which began a lifelong friendship. Several lines of evidence indicated that β -hydroxypropionate is an intermediate between β -alanine and acrylyl-CoA, which is then reduced to propionyl-CoA (2). Recent work indicates that the first step after formation of β -alanyl-CoA is direct deamination leading to acrylyl-CoA rather than a transamination leading to the formation of β -hydroxypropionate, thence to acrylyl-CoA (3).

Some time after I had settled into the Stadtman laboratory, I received a call from Konrad Bloch, who asked to meet with me when he was visiting the NIH campus. During lunch, he told me how his work on the aerobic formation of unsaturated fatty acids in eukaryotes had led to an interest in the formation of unsaturated fatty acids in anaerobic bacteria. He invited me to visit his laboratory at Harvard, which I did before the summer of 1959. After that visit, Konrad asked me to join his group as a

senior postdoctoral fellow, which I agreed to do in the autumn of that year after my work in the Stadtman laboratory was completed.

The Bloch laboratory had just moved into the newly built Conant laboratory building adjacent to the rest of the chemistry department. My laboratory mates were two graduate students, Marjorie Lindberg and Roger Childs. Further down the hall were Bill Lennarz (another new postdoctoral fellow), and Anne Norris (another graduate student), soon to be joined by Armand Fulco, a postdoctoral fellow. John Law's group occupied one of the adjacent laboratories. John was at the time an assistant professor appointed to complement the work of Konrad Bloch. At first I thought I would tackle a big problem, the morphogenesis of mitochondria, especially the formation or import of lipids. It soon became apparent that this effort was not going well, and Bloch was aware of this. We then discussed moving to a different problem. He asked: If eukaryotic organisms like yeast require molecular oxygen to desaturate saturated fatty acids, how do anaerobes produce unsaturated fatty acids (UFA)?² Indeed, do they even synthesize UFA? Since UFAs are required for the growth of *Clostridium butyricum* in the absence of biotin (4), we decided to study that organism. Twenty years later, my wife, Norah C. Johnston, and I found that the strain we worked with then and later should have been classified as *Clostridium beijerinckii*. Great efforts were taken

² The abbreviations used are: UFA, unsaturated fatty acid(s); ACP, acyl carrier protein; CFA, cyclopropane fatty acid(s); DGDAG, diglycosyldiacylglycerol; GAPsEtn, glycerolacetal of PlsEtn; Lm, *Listeria monocytogenes*; MGDAG, monoglycosyldiacylglycerol; PI, phosphatidylinositol; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific PLC; PlsEtn, plasmenylethanolamine; Ptd-*N*-methylEtn, phosphatidyl-*N*-monomethylethanolamine; PtdEtn, phosphatidylethanolamine, PtdSer, phosphatidylserine; PtdGro, phosphatidylglycerol; ts, temperature-sensitive; LPS, lipopolysaccharide; DAG, diacylglycerol; PKC, protein kinase C; LLO, listeriolysin O.

to ensure that the cultures had no molecular oxygen, and we quickly showed that *C. beijerinckii* produced UFA anaerobically. In a series of experiments with isotopically labeled short chain carboxylic acids, we found that those shorter than C12 gave rise to both saturated fatty acids and UFA, but longer chains were incorporated only into saturated fatty acids (5, 6). The pathway proposed, involving the introduction of the double bond at the C8 or C10 stage of chain elongation, is now recognized as the primary mechanism used by prokaryotes, even those that are strictly aerobic or, like *Escherichia coli*, facultatively anaerobic. These papers recently were recognized as classics by the *Journal of Biological Chemistry* (7).

During my time with Bloch, I discovered that the strain of *C. beijerinckii* we were using had an unusual phospho-*N*-methylethanolamine headgroup as a major lipid. In our attempts to obtain *N*-methylation of phosphatidylethanolamine (PtdEtn) in cell extracts using methyl-labeled SAM as the methyl donor, I found that radioactivity was incorporated into the phospholipid fraction, not into the headgroup, but rather into the hydrocarbon chains. Howard Zalkin, a postdoctoral fellow with John Law, was trying to obtain the formation of cyclopropane fatty acids (CFA) using cell extracts of various bacteria that had CFA. I quickly told him about my finding, and he confirmed that extracts of *C. beijerinckii* readily formed CFA and that the reaction probably took place with intact phospholipids (8).

Harvard Medical School

In 1961, after 2 years in the Bloch laboratory, I began to look for a faculty position. Fortunately, Bernard Davis, chair of microbiology at Harvard Medical School, was looking for a junior faculty member with experience in bacterial membranes and lipids. In the summer of 1962, I moved across the Charles River and started my own laboratory. I was very fortunate to have two postdoctoral fellows join me then: Nicole Baumann, from Paris, France, had had some postdoctoral experience at Columbia University, and Per-Otto Hagen, a Norwegian, had trained in Edinburgh, Scotland, and then with Morris Kates in Ottawa. Kates was one of the pre-eminent lipid biochemists in North America. A technician, Martha Ellis, completed my team funded through the NIH.

We immediately began work on the formation of plasmalogens in *C. beijerinckii*. Other researchers had shown that some anaerobic Gram-negative bacteria contained plasmalogens (1-alk-1'-enyl, 2-acyl phospholipids), and we had found them in *C. beijerinckii*, the first finding of ether lipids in a Gram-positive organism. In an early experiment, we examined the kinetics of labeling of the polar lipids of *C. beijerinckii* in a 20-liter carboy pulsed with 5 mCi of $^{32}\text{P}_i$. An experiment like that would horrify my colleagues today and would be closely monitored for radiation safety at Penn. We separated the lipid classes by preparative TLC and distinguished the plasmalogens, which are relatively alkali-stable and acid-labile, from the diacyl phospholipids. It was clear that the diacyl lipids were labeled first, followed by the plasmalogens after a 5–10-min lag, suggesting that the diacyl compounds might be precursors of the ether lipids. Similar results were obtained upon labeling with radioactive acetate (9, 10). Hagen provided an additional clue.

Long-chain aldehydes and long-chain fatty acids were readily incorporated into the alk-1-enyl chains of plasmalogens in *C. beijerinckii*. When labeled with tritium on C-1 of the aldehyde, some of the label was incorporated into the alk-1-enyl chains, indicating that there was direct incorporation in addition to the presumed incorporation by way of the *sn*-1 acyl chain (10).

Efforts to synthesize plasmalogens with cell-free extracts were not successful; however, we were able to show that the acylation of glycerol 3-phosphate is stimulated by the addition of reduced acyl carrier protein (ACP) generously provided by Konrad Bloch. Roy Vagelos and Gérard Ailhaud, at Washington University in St. Louis, were working along similar lines and observed that *E. coli* membrane preparations can catalyze the acylation of glycerol 3-phosphate with palmitoyl-CoA, yielding lysophosphatidic and phosphatidic acid, or with palmitoyl-ACP, leading to the formation of monopalmitin. Since our system appeared to require acyl-ACP and did not work with acyl-CoA, we decided to join forces. Roy sent Ailhaud, an experienced postdoctoral fellow, to Boston. He purified ACP from *C. beijerinckii* and prepared palmitoyl-ACP_{CB}. Acylation of glycerol 3-phosphate by membrane preparations required acyl-ACP and was inactive with palmitoyl-CoA. Thus, a clear distinction was drawn between eukaryotic organisms that use acyl-CoA in the formation of phosphatidic acid and prokaryotic organisms, such as *C. beijerinckii*, *E. coli*, and *Rhodobacter sphaeroides* (11), that use acyl-ACP. Later work by Charles Rock and colleagues has shown that in most prokaryotes, the acyl chain of acyl-ACP is transferred to P_i to form acyl-P, which then serves as the direct donor of acyl chains to glycerol 3-phosphate (12).

Penn Medical School

Around 1966, I had begun to receive invitations to visit other universities that were actively recruiting new faculty. One of these was the University of Pennsylvania, where Harold Ginsburg, co-author of an important textbook of microbiology with Bernard Davis, was chair of the microbiology department at the medical school. I decided that his offer of an associate professorship with tenure was the best fit for me and our growing family. But first, I had arranged for a sabbatical year in Bill Hayes' laboratory of microbial genetics at Hammersmith Hospital in London. There, I set out to isolate temperature-sensitive (ts) *E. coli* mutants with defects in lipid biosynthesis. A clever published technique allowed me to directly subject cells grown with $^{32}\text{P}_i$ to paper chromatography, to visualize the separated lipids by autoradiography. Of 500 ts mutants, several were promising. When I began work at Penn, Karim Hechemy and Seamus Rooney, postdoctoral fellows who arrived soon after my new laboratory opened, began to examine these ts mutants in detail.

We found that one of these mutants had a temperature-sensitive defect in the acylation of lysophosphatidic acid, the first indication that there are separate enzymes for this step and the initial acylation of glycerol 3-phosphate (13). Chromatographic analysis of lipids from another promising mutant revealed a new spot on autoradiographs, and Rooney's work revealed that it was a heptose-deficient form of lipopolysaccharide (LPS)

(14). Jane Koplow, a graduate student, separated inner and outer membranes of the mutant *E. coli* cells and found that the outer membrane had very low amounts of several proteins, now known as porins (15). Hiroshi Nikaido's laboratory at Berkeley had independently made a similar observation with heptose-deficient mutants of *Salmonella* (16). Forty years later, the connection between LPS biosynthesis and outer membrane protein export is much better understood (17).

Cyclopropane fatty acids

In a study of the fatty acids of *C. beijerinckii* with Charles Panos, we found that the CFA synthase was highly specific for the position of the double bond, with strong preference for *cis*-9–16:1 over the *cis*-7-isomer, even though both unsaturated 16:1 fatty acids were present. Some preference for *cis*-11 over *cis*-9–18:1 was also seen (18). Specificity was also observed for the CFA synthase of *E. coli*. A series of monounsaturated fatty acids were added to the growth medium of an unsaturated fatty acid auxotroph. As in *C. beijerinckii*, there was strong specificity for *cis*-9- and *cis*-11–16:1 and 18:1 isomers (19). These results suggested that the active site of CFA synthase is positioned to interact with double bonds in phospholipids at a certain distance from the ends of the acyl chains.

But what are the functions of CFA? It was known that they are formed in both Gram-positive and Gram-negative bacteria at the beginning of the stationary phase and that, with time, a significant proportion of unsaturated fatty acid is converted to CFA. We thought that they might have a protective effect for cells that had reached stationary phase and were forced to survive until conditions became conducive for growth resumption. John Harley, an M.D./Ph.D. student, approached this problem by subjecting an *E. coli* unsaturated fatty acid auxotroph, fed a series of monounsaturated, polyunsaturated, and cyclopropane fatty acids, to hyperbaric oxygen. He found that cells grown on a C19 cyclopropane fatty acid (9,10-methyleneoctadecanoate) were more resistant to hyperbaric oxygen killing than cells grown on oleate (*cis*-18:1), which in turn were more resistant than cells grown on C18 polyunsaturated fatty acids. He further showed that treatment with hyperbaric oxygen did not result in oxidation of cellular monounsaturated fatty acids, leading to the conclusion that CFA provide a barrier rather than resistance to oxidation of the membrane fatty acids (20).

The barrier function of CFA was further emphasized by the sensitivity of *afa* mutants to acid shock (21). Recent work with a *afa* mutant of *Salmonella* Typhimurium found that the mutant was more sensitive than the WT to acidic conditions, H₂O₂, and the protonophore carbonyl cyanide 3-chlorophenylhydrazone. There was increased sensitivity to oxidative conditions in murine macrophages and attenuation of virulence in a murine model of infection.³ In *Mycobacterium tuberculosis*, cyclopropanation of long-chain mycolic acids is important for resistance to oxidative stress and virulence in animals studies (23). It appears that CFA have evolved to protect bacteria against stresses to help ensure long-term survival and, in the case of pathogenic bacteria, to help circumvent host defenses.

Anaerobic plasmalogen biosynthesis

From the work of several laboratories in the early 1970s, it became clear that plasmalogen synthesis in animal tissues requires molecular oxygen (24); thus, the mechanism in anaerobic bacteria has to be fundamentally different. In animal tissues, a saturated long-chain ether is desaturated to form the 1-*O*-alk-1-enyl ether, an oxygen-dependent step, reminiscent of the desaturation of saturated fatty acids. Our earlier work with proliferating clostridial cells led us to think that the alk-1-enyl ether was formed from the *sn*-1-acyl chain. We examined all of the enzymatic steps from phosphatidic acid to PtdEtn and phosphatidylglycerol (PtdGro) and found no hint of ether lipid formation. Yosuke Koga, visiting from Kitakyushu, Japan, found that, when phosphatidylserine (PtdSer) decarboxylase was inhibited with hydroxylamine in proliferating cells of *C. butyricum*, PtdSer accumulated, as expected, and was almost entirely in the diacyl form. When inhibition was released, PtdEtn was rapidly made, followed by plasmenylethanolamine (PlsEtn), and subsequently by the glycerolacetal of PlsEtn (Fig. 3). Again, the evidence pointed to derivation of the plasmalogen from the corresponding diacyl lipid (25). More recently, we observed the same series of reactions by MS. We incubated proliferating *C. beijerinckii* with di-17:0-PtdSer and observed the formation of di-17:0-PtdEtn, di-17:0-Ptd-*N*-methylEtn, and the plasmalogens di-17:0-PlsEtn and Pls-*N*-methyl Etn, also with two 17:0 chains.⁴ These results are still preliminary, since the experimental conditions have been difficult to reproduce (26).

Plasmalogen biophysics

Although some bacteria, including strains of *Megasphaera elsdenii* and *Clostridium tetani*, survive and grow well in the laboratory in the absence of plasmalogens (27, 28), the synthesis of plasmalogens is critical for the development of the human nervous, heart, and skeletal systems (29). Gopal Khuller joined the laboratory in the early 1970s after initial postdoctoral training with Patrick Brennan. We thought that studies on the physical chemistry of plasmalogen-rich lipids and membranes from *C. butyricum* might provide insights into the special properties of these ether lipids. Remembering that fatty acid biosynthesis in *C. beijerinckii* depends on the presence of biotin in the growth medium, we developed semisynthetic media that were devoid of biotin. As expected, growth required the addition of an unsaturated, cyclopropane or branched-chain fatty acid. The same strategy worked with *C. butyricum*, which had the advantage of having the ethanolamine, but not the mixtures of ethanolamine and *N*-monomethylethanolamine, headgroups found in *C. beijerinckii*.

When *C. butyricum* grew without biotin in media supplemented with oleic acid, both the acyl chains and the alk-1-enyl chains were highly enriched in 18:1; however, cells also formed a 19-carbon cyclopropane fatty acid from 18:1, so the chains were not homogeneous. This was solved by growing cells with elaidic acid (*trans*-9–18:1), which is not a substrate for cyclopropane synthase. Dielaidoyl PlsEtn underwent the transition

³ J. E. Karlinsey, A. M. Fung, N. C. Johnston, H. Goldfine, S. J. Libby, and F. C. Fang, unpublished data.

⁴ B. Tian, P. Axelsen, and H. Goldfine, unpublished observations.

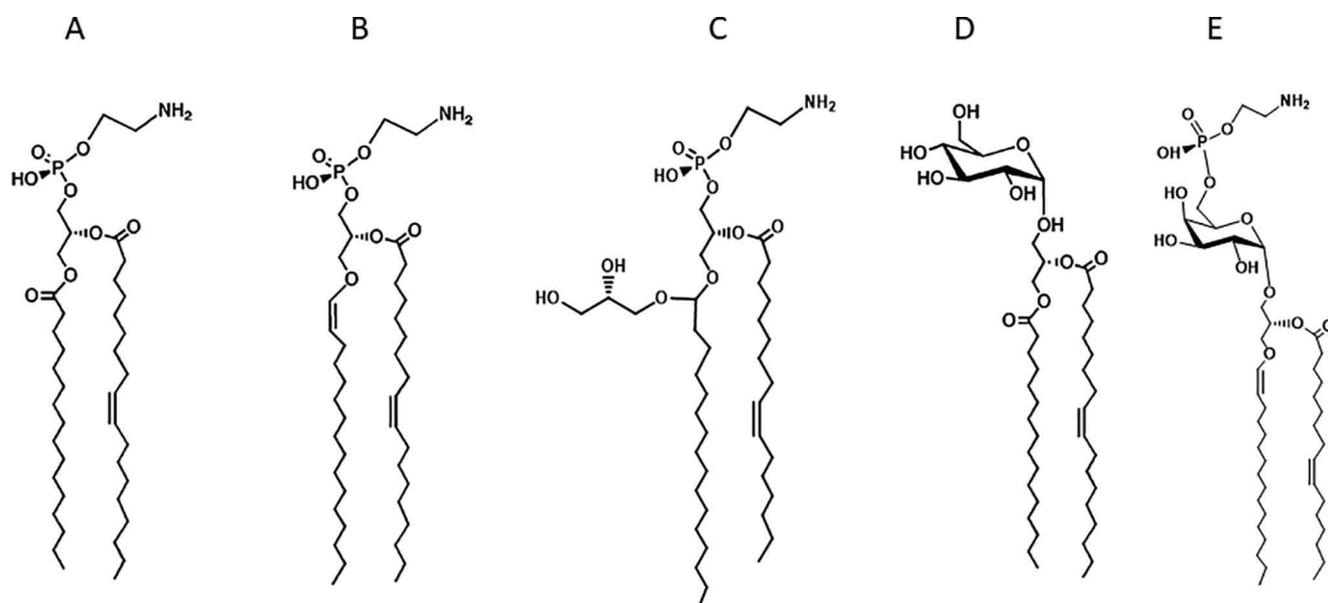


Figure 3. Some lipids found in various species of *Clostridium*. A, phosphatidylethanolamine; B, plasmenylethanolamine; C, glycerolacetal of plasmenylethanolamine; D, hexosyldiacylglycerol; E, ethanolamine-P-hexosyldiradylglycerol.

from the gel to liquid-crystalline phase at temperatures $\sim 5^\circ\text{C}$ lower than those for the corresponding diacyl lipid. The glycerolacetal of PlsEtn (GAPsEtn) showed a remarkably large hysteresis of $12.5\text{--}16^\circ\text{C}$ on heating and cooling. Cells fed oleic but not elaidic acid revealed an interesting phenomenon: The relative amounts of PtdEtn and PlsEtn decreased markedly and were largely replaced with GAPsEtn (Fig. 3). The significance of this change became apparent several years later (see below).

The ability to incorporate fatty acids into the diacyl phospholipids and plasmalogens of both *C. butyricum* and *C. beijerinckii* in the absence of biotin provided another means to probe their structures. Joachim Seelig at the Biocenter in Basel, Switzerland, provided palmitic and oleic acids labeled with deuterium at various positions of the chains, which we incorporated into the growth medium. NMR studies of the purified lipids revealed unusual quadrupole splittings at the C-2 position of the *sn*-2 chain of PlsEtn, which indicated that this chain is perpendicular to the bilayer at all segments of the chain. This is distinct from the known conformation of PtdEtn, in which the first two carbons of the *sn*-2 chain are nearly parallel to the bilayer surface. The quadrupole splittings obtained at the C-2 segment of deuterated oleic acid at the *sn*-1 position of the plasmalogens indicated that the double bond between carbons 1 and 2 is perpendicular to the bilayer surface (30). Similar findings with regard to the conformation of the *sn*-2 chain were made with deuterated semisynthetic ethanolamine plasmalogens (31).

In the early 1980s, we became aware of the remarkable work being done at Umeå University in Sweden on the variation of lipid composition in *Acholeplasma laidlawii*, a natural fatty acid and sterol auxotroph. When these bacteria were grown with mixtures of oleic and palmitic acid (16:0), there was considerable variation in the amounts of major polar lipids, the most significant of which, from our perspective, was the replacement of monoglucosyldiacylglycerol (MGDAG) (Fig. 3) by diglucosyldiacylglycerol (DGDAG) as the degree of unsatu-

ration of the total lipids became enriched in oleate chains (reviewed in Ref. 32). Referring to the lipid shape concept of Jacob N. Israelachvili *et al.* (33), which stated that lipids like DGDAG, phosphatidylcholine, and others that are essentially cylindrical in shape could readily form bilayers, whereas lipids like MGDAG and PtdEtn, which are more conical with relatively small headgroups and a tendency to hydrogen bond at the headgroup level, are prone to forming nonbilayer assemblies such as cubic and reversed hexagonal phase (HII), the group at Umeå University believed that the changes they observed in *A. laidlawii* promote the stability of the bilayer arrangement in the cell membranes.

In the late 1970s, my wife Norah, who had worked on honey bee pheromones at the National Institute for Medical Research in London as a graduate student and did postdoctoral training with John Law and later with Konrad Bloch at Harvard, joined the laboratory as a research associate. We decided to test the hypothesis that GAPsEtn could serve the same function in *C. butyricum* as DGDAG does in *A. laidlawii*. We built models of GAPsEtn with the extra glycerol pointing toward the aqueous phase. Clearly, with its glycerol hydration shell, GAPsEtn would have a much larger polar headgroup than PtdEtn and PlsEtn (Fig. 3C). The latter was shown to have an even greater propensity to form nonlamellar phases than PtdEtn when enriched in unsaturated chains (34). We tested this hypothesis by growing *C. butyricum* in the absence of biotin with mixtures of oleic and palmitic acids, and the results were gratifying. At $>40\%$ oleic acid, much of the PtdEtn/PlsEtn was replaced by GAPsEtn (35). We also showed that PtdEtn, PlsEtn, and GAPsEtn were concentrated in the outer leaflet of the cell membrane. The next question we asked was: Does GAPsEtn stabilize the bilayer phase? We used differential scanning calorimetry, ^{31}P NMR, and X-ray diffraction, in collaboration with Graham Shipley at Boston University, to reveal that GAPsEtn stabilizes the bilayer arrangement when added to oleate-enriched PtdEtn/PlsEtn mixtures (36).

As part of this work, we grew *C. butyricum* and *C. beijerinckii* with various fatty acids in the absence of biotin and found that lipid shape did indeed affect lipid composition. In short, the higher the tendency of PtdEtn/PlsEtn to promote formation of a nonbilayer phase, the greater is the replacement of those lipids with GAPsEtn, which helps stabilize the bilayer phase (37). Similarly, when solvents such as cyclohexane or *n*-octanol, which perturb the bilayer arrangement, were added to the elaidate-supplemented media, the ratio of GAPsE to PtdEtn plus PlsEtn was significantly increased. In *A. laidlawii*, the ratios of DGDAG to MGDAG similarly responded to solvent addition to protect the bilayer arrangement (38).

Our results with lipid structures very different from those found in *A. laidlawii* added credence to the theory that biological membranes contain mixtures of bilayer-forming and nonbilayer-forming lipids to achieve a balance of forces. This concept has been strengthened by work with other organisms, including *E. coli*, as discussed by William Dowhan in his Reflections article (39). As proposed by Lindblom and colleagues (40, 41), cells appear to live within a narrow window where their membranes are in a physical state between the gel phase and the formation of nonbilayer aggregates. Although the concept of regulation of membrane fluidity has been widely accepted and can be found in almost every textbook, the regulation of an equilibrium between lipids forming bilayers or nonlamellar phases, while equally important, is not as well-known or even understood. Why has this paradigm, if I may call it that, been so neglected? This is a challenge for historians of science!

Listeria monocytogenes

The next advances in understanding how the synthesis of GAPsEtn is regulated we believed would require identification of the genes and enzymes needed for the formation of the glycerol acetals of plasmalogens. These lipids have been found only in clostridia related to solvent-producing species, such as *C. beijerinckii* and *C. acetobutylicum*, and in the late 1980s, methods for unraveling the genetics of clostridia were not well-developed. Understandably, funding for this next stage was difficult to obtain. In 1988, our department had the good fortune to recruit Daniel (Dan) Portnoy, a promising young microbiologist who was studying the pathogenesis of *L. monocytogenes* (Lm). The pathogenic potential of Lm, a food-borne bacterium, was increasingly being recognized, because Lm was found in many foods, and listeriosis, although rare, had a high mortality rate, especially in pregnant women and the elderly.

Dan told me there was evidence that Lm produces phospholipases and suggested that our experience with lipids should enable us to shed some light on this problem. Andrew Camilli, a graduate student with Dan, carried out *in silico* analysis and told us that an ORF reported by Pascale Cossart's group at the Pasteur Institute in Paris had amino acid sequence homologies to a phosphatidylinositol-specific phospholipase C (PI-PLC) previously studied in *Bacillus cereus* and *Bacillus thuringiensis*. We confirmed that Lm does indeed secrete a PI-PLC, and, with these preliminary data, Dan and I were able to obtain funding for a proposal to study the phospholipases of Lm. We established conditions for obtaining good yields of PI-PLC from growth supernatants and with old-fashioned ion-exchange and

gel-filtration chromatography were able to obtain highly purified PI-PLC (42). As expected, it was specific for PI and had no activity on phosphorylated forms of PI. We also noted that, unlike the classical form from *B. cereus*, it appeared to have only weak activity on a glycosyl-PI-anchored protein. With the help of an undergraduate student from Bryn Mawr College, Ami Gandhi, we extended these findings to other GPI-anchored proteins (43).

In addition to PI-PLC, Lm secretes a broad-range PLC with activity on all of the major membrane lipids of mammalian cells (44, 45). Deletion mutations of *plcA*, the gene for PI-PLC, and *plcB*, the gene for the broad-range PLC, individually have only small effects on mouse virulence; however, a double mutant exhibits a 500-fold increase in mouse LD₅₀. Clearly, these phospholipases fulfill overlapping roles in the intracellular growth and spread of Lm (46). What these were became the focus of our joint efforts with the Portnoy laboratory. Trudy Bannam and Wolf Zückert, who were postdoctoral fellows, showed that the biological activities of these phospholipases are completely dependent on their ability to cleave their respective substrates (47, 48), which shot down our previous hypothesis that PI-PLC_{Lm} disrupted membranes without lipid hydrolysis (49). Substitution of *B. cereus* PC-PLC, which has lower activity on sphingomyelin than broad-range PLC (*PlcB*) from Lm, resulted in reduced ability of Lm to spread from cell to cell, an important function of the Lm enzyme (48).

Given that PI-PLC_{Lm} did not release GPI-anchored proteins, what function does it fulfill? Lm is a facultative intracellular pathogen and is able to escape from the primary phagocytic vesicle of mammalian macrophages, grow in the cytosol, and spread from cell to cell. It promotes the polymerization of host actin, which propels it through the host cell's cytoplasm and into adjacent cells (50). Camilli *et al.* (51) had shown that PI-PLC deletion mutants were defective in escape from the phagosome. We considered the possibility that, through cleavage of host PI and generation of diacylglycerol (DAG), a well-known lipid second messenger, Lm could be manipulating normal host processes for its own ends. Attempts to study the generation of DAG by conventional means showed that this lipid accumulates slowly in infected macrophage-like cells, but chemical measurements did not reveal the kinetics of this process (46). At this time, Sandra Wadsworth, an experienced cell biologist, joined our group and began to examine the possibility that changes in intracellular calcium levels, in association with increases in DAG generated by Lm PI-PLC, would activate host PI-PLCs and classical protein kinase C (PKC). She showed that intracellular calcium levels in murine macrophage-like J774 cells changed as early as 1 min in cells infected with washed suspensions of Lm. These changes, which occurred before the bacteria were internalized, were completely dependent on listeriolysin O (LLO) and partially dependent on the Lm phospholipases. Each phospholipase contributed in different ways. The most important, in terms of escape from the primary vacuole, was PI-PLC, without which the early calcium spike was not seen and later spikes were either absent or diminished. It appeared that the early events of attachment and internalization of Lm to these cells were controlled by the actions of PI-PLC. Whereas there was a delay of internalization of WT Lm, a mutant in

PI-PLC was taken up much more rapidly. Surprisingly, it appeared that the actions of these virulence proteins slowed the entry of Lm into macrophage-like cells (52). Were they able to affect their eventual fate in the host, prior to entry?

Calcium elevation and the formation of diacylglycerol are known to activate PKC. Both LLO and PI-PLC were found to be required for rapid translocation of PKC δ to the cell periphery and for association of PKC β II to early endosomes. Inhibition of PKC β resulted in more rapid uptake of Lm into cells and decreased escape from the phagocytic vacuole, mimicking the effects of deletion of the gene for PI-PLC (53). It appears that PI-PLC enters cells through pores created by LLO and rapidly modifies the host to delay entry and promote escape from the phagocytic vacuole. The importance of PKC β was further emphasized by studies of macrophages from PKC β knockout mice. Disappointingly, WT Lm escaped from the phagosome of macrophages from PKC β knockout mice as well as it did from macrophages from WT mice. However, in macrophages from the mutant mice, deletion of the gene for PI-PLC no longer resulted in a defect in escape from phagosomes. Instead, a mutant in the broad-range phospholipase now showed a defect in escape not seen in infections WT macrophages. These findings strongly support the hypothesis that PI-PLC promotes escape from immune surveillance through mobilization of host PKC β (54).

An understanding of the lack of activity of Lm PI-PLC on GPI-anchored proteins came with the solution of its crystal structure by Heinz and colleagues (55). The protein has a $(\beta/\alpha)_8$ -barrel structure like that of PI-PLC from *B. cereus*. However, the listeria protein lacks the Vb β -strand, which is important for binding to the glycan linker of GPI-anchored proteins. Thus, when PI-PLC comes into contact with the host cell from the extracellular space, it interacts only weakly with the surface, thus enabling it to enter the host cell rapidly and to interact with host PI. When *B. cereus* or *B. anthracis* PI-PLC, which is very similar to that from *B. cereus*, was expressed in Lm, the ability to escape from the phagocytic vacuole and spread from one cell to another was greatly diminished, as was virulence in mice (56). Deletion of the Vb β -strand from *B. cereus* PI-PLC led to loss of its ability to cleave glycosylphosphatidylinositol-anchored proteins and improved its ability to replace Lm PI-PLC, which resulted in an increase in mouse virulence. It appears that loss of the Vb β -strand of PI-PLC is an evolutionary adaptation to an intracellular lifestyle (57).

Return to anaerobes

I formally retired in 2011 after 49 years of running my research laboratory first at Harvard Medical School and then in the Perelman School of Medicine at Penn. However, the biosynthesis of plasmalogens in anaerobes represented a gap in our knowledge of this important pathway, which we now know is conserved in most, if not all, clostridia and in other anaerobic species. We decided to pursue a genomic approach to this problem; however, none of the genes we selected for testing proved to be required for plasmalogen biosynthesis. Part of this project was to examine the lipids of clostridia of which the entire genome had been sequenced. Among the first was *C. tetani*, the cause of human tetanus, a serious and sometimes fatal disease.

Analysis of the lipids of the parent of the genome-sequenced strain revealed that it did not have plasmalogens; however, examination of several other strains revealed that all contained plasmalogens in addition to the diacyl forms. In all strains, we observed a new derivative of *N*-acetylglucosaminyl diradylglycerol, which has phosphoethanolamine attached to the 6'-position of the sugar (Fig. 3). Ethanolamine-P-modified glycosyldiradylglycerols were also found in *C. acetobutylicum*, in which they also appear to function in parallel with diglycosyldiradylglycerol to stabilize the bilayer arrangement in the presence of octanol (58). High-resolution MS revealed that phosphatidic acid and phosphatidylserine in *C. tetani* and *Clostridium novyi* were almost entirely in the diacyl form, consistent with our hypothesis that plasmalogens are formed at a late stage of phospholipid assembly in anaerobes (28, 59).

After the untimely death of my friend and collaborator Chris Raetz, we were fortunate to be able to continue this work with his colleague Ziqiang Guan, an accomplished mass spectroscopist. Since then, we have studied the lipidomes of *C. novyi*, *Clostridium psychrophilum*, *Clostridium botulinum*, *Clostridium fallax*, *Clostridium cadaveris* (for references to lipids of other clostridia, see Ref. 22), and *Clostridium phytofermentans*.⁵ All of the species of *Clostridium* (*sensu stricto*) that we and others have studied are now known to contain plasmalogens.

These studies have shown that *Clostridium* (*sensu stricto*), which has over 35 species, has considerable diversity of lipids, some of which are characteristic of the branch of the clostridial tree to which they belong. For example, we have found that glycerol acetals of plasmalogens are present only in species related to *C. butyricum*, including *C. botulinum* Group II. *C. novyi* and related strains of *C. botulinum* Group III are characterized by the presence of aminoacyl-phosphatidylglycerols, with lysine and alanine being the predominant amino acids. *Clostridium sporogenes* and *C. botulinum* Group I have an ethanolamine-P-*N*-acetylglucosaminyl-diradylglycerol similar to that identified in *C. tetani*, which is related. With modern methods of LC/MS, the study of clostridial lipidomes may yet reveal other significant lipids that have evolved for the fitness of individual species.

Coda

I have been very fortunate in many ways. First, by having parents who were supportive of my progression from high school to college and graduate school, in the face of their declining health and economic stability. Second, by living in New York City, which provided *free* college education to thousands of students of limited means. Third, by having teachers who stimulated my interest in biochemistry and encouraged me to do graduate work in that field. I was also fortunate in my choice of postdoctoral mentors, Earl Stadtman and Konrad Bloch, who provided excellent research environments along with enough freedom to develop my own paths into research. Graduate students should note that the importance of these choices in preparing for a career in research cannot be overemphasized.

My wife Norah has provided steadfast support both at home and in the laboratory. Her training in chemistry has been crucial

⁵ Z. Guan and H. Goldfine, unpublished observations.

to our studies on bacterial lipid structures and functions. I wrote these reflections at the urging of Herbert Tabor, who at age 101 continues to be an inspiration to generations of biochemists. Last, I have to express my appreciation to colleagues who have been essential for the development of our research program. I will not name them here: They can be found in the text and in the list of references. It has been a long, sometimes bumpy, and fulfilling life in science, which thankfully has not ended.

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