Technical News Feature

Radiochromatographic Exploration

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Andrew A. Benson was the recipient of the 1987 Supelco AOCS Research Award presented in May at the 1987 AOCS annual meeting in New Orleans, Louisiana. The following is the award address given by Benson. In it, he focused on radiochromatographic exploration, involving examination of chromatographically separated radioactive products of cell or tissue metabolism. Investigation of unanticipated metabolites revealed in radioautographs of two-dimensional paper chromatograms has led to delineation of new biochemical pathways and to recognition of novel amphipathic such lipids. Identification and possible function of such lipids containing phosphorus, sulfur, arsenic and antimony are reviewed.

To see and understand the unkown fascinates and gives us a feeling of satisfaction, whether it be a novel ascent in the Himalaya, a visit to the moon or to the sea bottom. Nature still has plenty of unknown regions to explore. Each requires special training, tools and a concept of the goal. I have been fascinated by the power of the radiochromatographic method and the metabolic systems it has revealed.

Metabolic exploration offers challenges and risks which explorers have always known. It exposes the explorer to the dread and uncertainty of error or success. So often the explorer cannot bear to recall or to write of his countless steps in the wrong direction. In the light of the ultimate beauty and simplicity of nature, the explorer cannot bear to expose his human frailty and unsuccessful gropings toward a goal which lacks

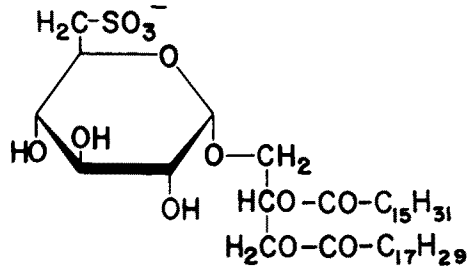


FIG. 2. Structure of the plant sulfolipid. Note unique fatty ester locations.

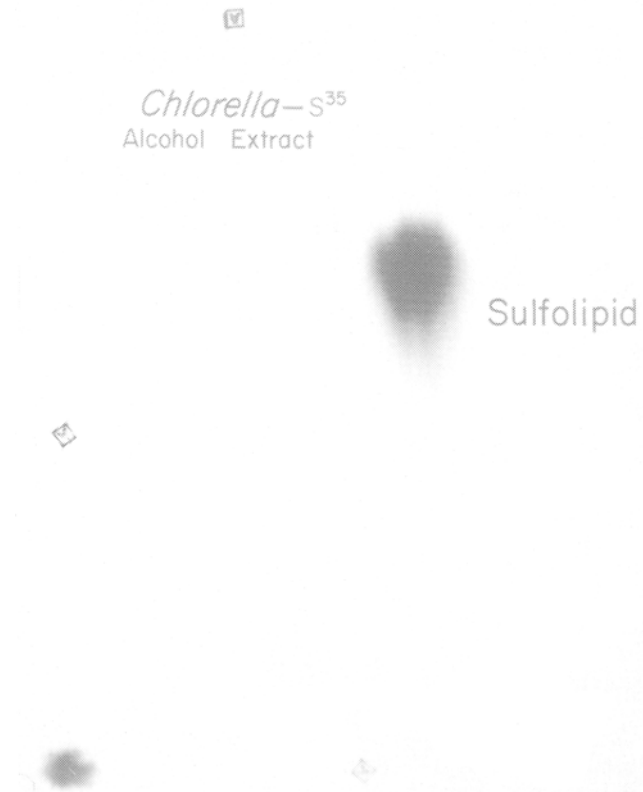
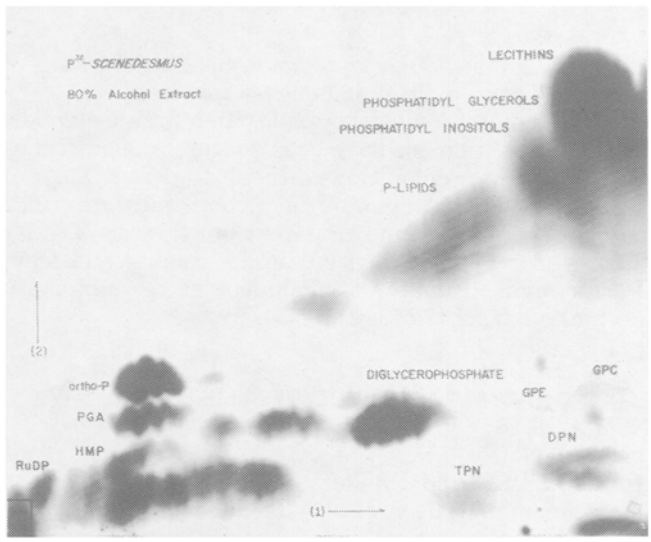


FIG. 1. Radiogram of ^{35}S-labeled components, largely sulfolipid, of the green alga *Chlorella pyrenoidosa*.



***Scenedesmus* D_3.**

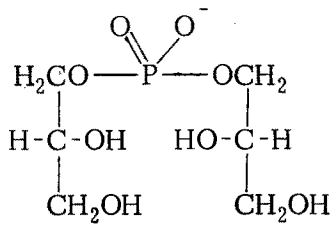


FIG. 4. Diglycerophosphate, GPG.

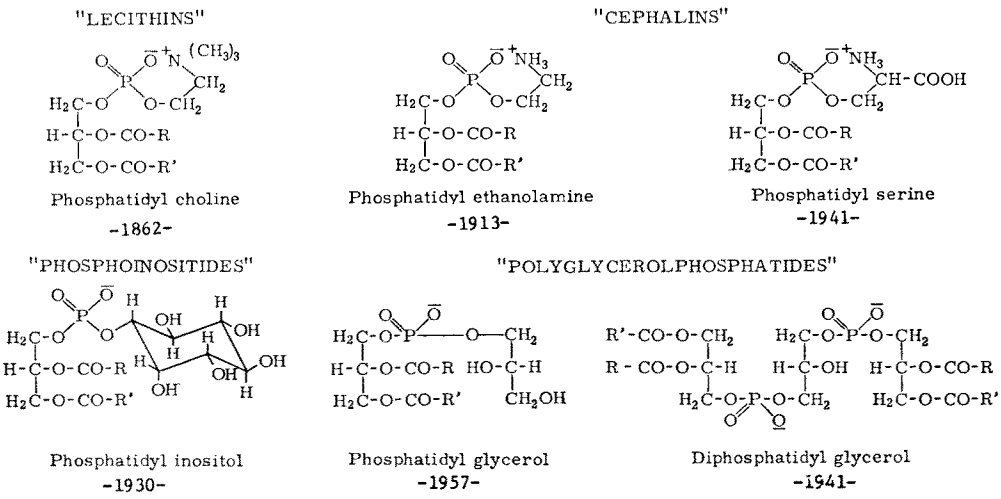


FIG. 5. The glycerolphosphatides.

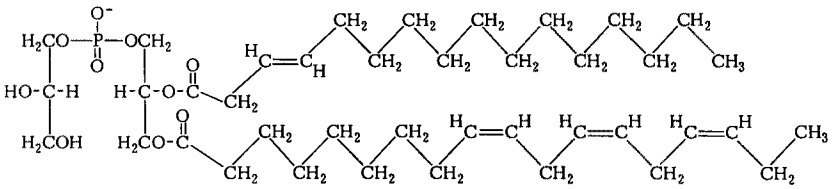


FIG. 6. Phosphatidylglycerol of chloroplasts.

reality until it is achieved. To look back at the path of discovery is too painful; it is never revealed.

I have enjoyed molecular exploration for novel lipids. From time to time, we have been successful, sometimes as the result of premonitions difficult to define. Most of our work seems to answer the wrong questions. The right questions become apparent only later. I offer three examples; each elicited similar doubts and fears of error, and, ultimate astonishment at the simplicity and molecular logic of nature.

PAPER CHROMATOGRAPHIC RESOLUTION OF MIXTURES

Modern HPLC and capillary GLC technology, whose development has been clearly facilitated by the sponsors of the Supelco AOCS Research Award, possess remarkable resolving power for mixtures of similar compounds, fatty esters, silylated derivatives, triglycerides or wax esters. But when the subjects of the investigation are tracer amount unknowns which can be mixtures of protein, polysaccharide, amino acids, phospholipids or even hydrocarbons, the expensive machinery becomes confused and we must go back to liquid-liquid partition. My experience with the first C-14 and tritium when working with Sam Ruben and Martin Kamen established the partition methodology basic to separation of tracer amounts of biochemical substances.

Unlike adsorption, partition (distribution) provides valid separations even at tracer-level concentrations.

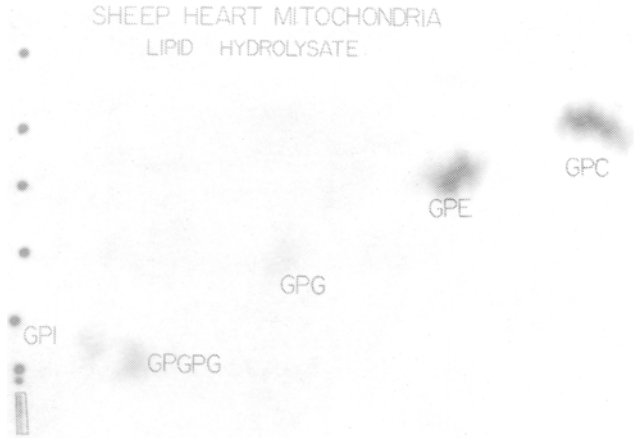


FIG. 7. Radioautograph of neutron-activated chromatogram of deacylated sheep heart mitochondrial lipids. Note 1 μg phosphorus samples applied near edges of developed chromatogram.

In paper chromatography, separations result from partition between a moving organic phase and a stationary aqueous phase adsorbed on the cellulose. As the solvent travels, it loses water and water-soluble components to the dry paper; the solvent composition is actually a gradient beginning with high water content and changing to one with low water and increased concentration of the more hydrophobic component. Our n-butanol-propionic acid system (1) loses water and

propionic acid to the paper phase as it travels; actually, even the acidity changes as it travels. Thus, the system separates acidic and basic compounds in addition to resolution on the basis of size or molecular weight. Series of a dozen polyglucoses separate on the same sheet which resolves a number of phospholipids and triglycerides. The insoluble proteins remain on the origin. An X-ray film exposed to this sheet records everything in the original system. It is an ideal approach for an unknown mixture of metabolic products.

The HPLC or GLC enthusiasts may say their separations are faster. That depends on one's point of view. They do learn the result sooner, but I have seen so many colleagues standing patiently glued to their GLC recorder pen for hours. Making a paper chromatogram and developing the radioautograph actually consumes only a few minutes. There is no limit to the number of projects which can be proceeding at the same time.

NATURE'S GREAT DETERGENT, THE PLANT SULFOLIPID

In the course of looking for a disulfide intermediate in photosynthesis with Melvin Calvin (2), we found in algae a tremendous amount of a single radiolabeled lipid (Fig. 1). It was not the lipoic acid derivative we sought. So we dropped work on this lipid until I resumed the study at Penn State with Bob Wiser and Helmut Daniel (3). Its amphipathic properties led to its glycolipid structure, which I guessed must be a sulfate ester of the plant galactolipid. Daniel, being a fine young organic chemist from the University of Munich, wasn't constrained by biochemical dogma. He insisted it was a sulfonic acid of a 6-deoxysugar and proceeded to prove it (Fig. 2). We had to agree, but it was frightening. Nature had had no such sulfonic acids in her sugar bin. Synthesis by Masateru Miyano (4), isolation by Marius Lepage (5,6) and finally X-ray crystallographic structure determination by Yoshi Okaya (7) laid the problem to rest. The fact that the inviolable specific crystalline enzyme, beta galactosidase, hydrolyzed the glycoside (8) made it, obviously, a betagalactoside. The enzyme made a mistake; it is an alpha glucoside, more accurately 1-O-(α-6-sulfoquinovosyl)-D-glycerol (quinovose being 6-deoxy-D-glucose).

By 1963, the sulfolipid was isolated (9) and characterized, but its involvement in plant metabolism has remained a mystery. As a sulfonic acid, it has affinities for sodium ion and calcium ion very different from those of the glycerolphosphatides. It could play a unique role in selective ion binding or transport. We did find an intriguing collection of sulfonic acid analogs of the well-known phosphorylated intermediates, even one which had properties of a nucleoside diphosphate sulfosugar (10). But that's as far as it has gone. Since then, many excellent research groups have tried to go further but have failed. It seems to me that research without a vision of some metabolic or structural role for this lipid is most likely futile. So, for the present, forget it. Let's hope that soon, someone will get a delightfully bright idea.

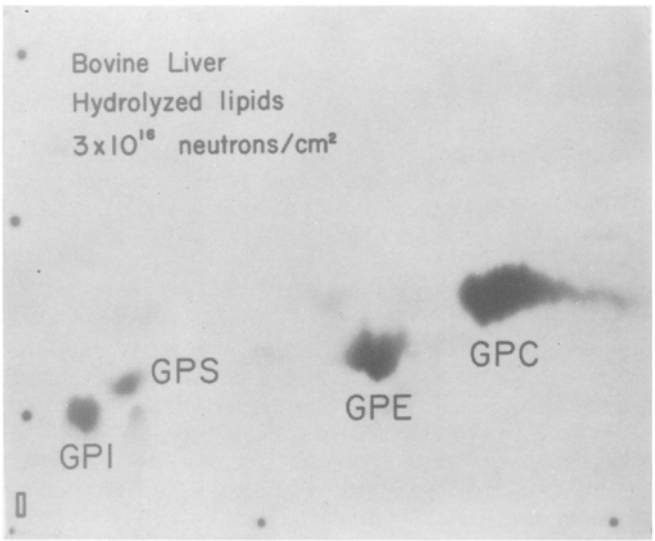


FIG. 8. Radiogram of neutron-activated deacylated lipids of bovine liver.

PHOSPHATIDYLGLYCEROL

It seems not long ago that Nick Pelick and I were trying to label algae with C-14 to obtain radioactive lipids. I didn't do very well but did discover tht Tygon tubing in roller pumps was the source of considerable leakage. Anyhow, we had a lot of fun trying. It was a delight to work with Nick Pelick, a remarkable storehouse of energy and expertise in lipidology. His ultimate success was inevitable.

In that Penn State laboratory, we had just discovered phosphatidyl glycerol. Although we had not yet beome aware of its global importance in bacterial membranes, we had learned much of its importance for plant chloroplasts.

The search had been engendered by appearance of an unknown phosphate ester in radiochromatograms of P^{32}-labeled algae (Fig. 3), spinach and other plants. The unknown appeared in large quantity and separated easily from the sugar phosphates. By acid treatment, it was converted to a more polar phosphate ester, sometimes with intermediate formation of a much more labile compound. At that time, Bunji Maruo, then assistant professor in the University of Tokyo's Institute of Applied Microbiology, joined our laboratory and tack-

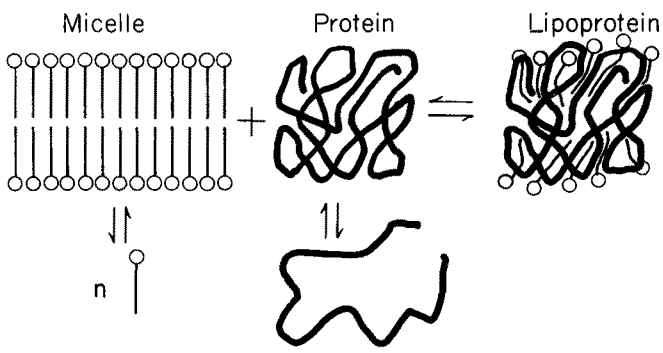


FIG. 9. Hydrophobic interactions of amphipathic lipids and of protein.

led the unknown. In a radiochromatogram of a plant extract, we see everything: insoluble materials, ionic compounds, sugars, amphipathic lipids and hydrophobic lipids. It was clear the unknown was an ubiquitous component of plants. Maruo succeeded in deducing the identity of the hydrolysis product as glycerophosphate, a first step toward its relationship to lipids. With C^{14} labeling of the unknown, we obtained indications that glycerol itself was the other component. To assign its structure simply as diglycerophosphate (Fig. 4) was neither easy nor obvious (11). Note concerning glycerophosphate: By considering the phosphate ester of natural glycerophosphate on C-1, the glycerol assumes a "D-" configuration which facilitates compact and definitive structural formulae. My discussions with H.O.L. Fischer and leading carbohydrate chemists in the period 1956-1960 received favorable responses. Unfortunately, contemporary nomenclatural committees failed to recognize obvious simplicity and its perpetual importance for students of lipid biochemistry.

It was at the time that Alexander Todd and his colleagues at Cambridge had finished determining the phosphate diester linkages in DNA and RNA and their mechanism of acid hydrolysis. The acid-catalyzed transesterification led to a cyclic ester intermediate (12) and its subsequent hydrolysis, reactions which had previously impeded understanding of RNA structure.

Diglycerophosphate looked like a skeleton of a phospholipid more simple than lecithin or cephalin (Fig. 5). We deacylated the P^{32} lipids of plants and, in each case, found about 20% of diglycerophosphate, GPG. Even rat liver contained a phosphatide producing GPG. How could such a simple lipid, containing only phosphate, glycerol and fatty acid, have been overlooked in the 100 years of phospholipid study? The structure was too simple. It was frightening to send off the manuscript to Biochimica et Biophysica Acta (13). We could so easily be missing something obvious. As it turned out, the rest of the world had missed something obvious. Just last October in Tokyo, Bunji Maruo presented me with a pound of synthetic phosphatidyl glycerol. It is one of the easiest lipids to produce by transphosphatidylation (14) from choline of lecithin to glycerol. Phosphatidyl glycerol is a chloroplast lipid (Fig. 6). Its real role, though, is still unclear. Its 25% Δ3, tr-hexadecenoic ester in the 2-position is unique in nature but still not explained.

NEUTRON ACTIVATION CHROMATOGRAPHY

Radiochromatography is not limited to living systems such as a weanling rat which may be labeled with ^{32}P for analysis of its phosphorus compounds. We can neutron-activate many elements such as ^{31}P and ^{32}S *after* separation of their compounds on paper chromatograms (or TLC). Hence, we obtained useful radiograms of phospholipids from sheep heart mitochondria, (Fig. 7), bovine liver (Fig. 8) and serum lipoproteins. With adjacent one-microgram phosphorus standards, the method offers both qualitative and quantitative sensitivity (15). With appropriate scintillation detectors and gamma spectrometry, neutron activation chromatography becomes feasible for many elements. By

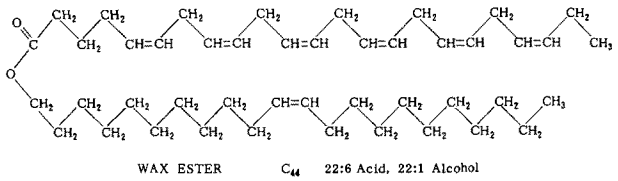


FIG. 10. Marine wax ester structure.

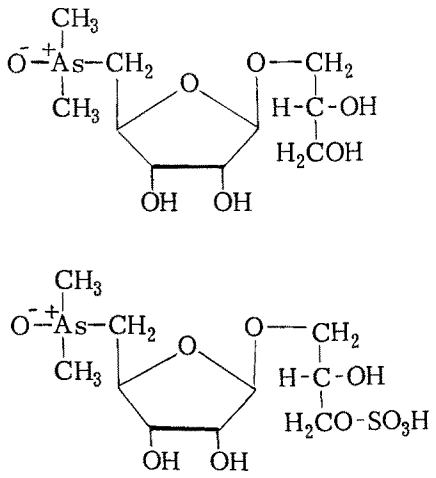


FIG. 11. The algal arsenoribosides.

adding a bromine in the form of bromophenacyl esters or other brominated derivatives, sugars and amino acids can be analyzed quantitatively (16). Chromatographic separation prior to activation vastly broadens the analytical usefulness of neutron activation analysis.

FUNCTIONS OF AMPHIPATHIC LIPIDS

Like Ira Gershwin's "Sportin' Life" said, "Lipids is two-faced"—amphipathic from the Greek, meaning both types hydrophilic and hydrophobic in character. We know they associate in monolayers at a water-air interface and in bilayers in artificial and natural cell membranes. But we know frightfully little of how the hydrophobic fatty acid chains might interact with the amphipathic proteins of cell membranes. We know that certain lipids interact favorably with certain membrane proteins. No one, however, dares describe the nature of such interaction except in vague "black-box" terms.

Twenty years ago, Tae Ji and I examined the stoichiometry of chloroplast lamellar lipid-protein interaction (17). The results may not be real, but they were interesting. Measuring the number of single hydrophobic-chained lipids like chlorophyll or radioactive sodium dodecyl sulfate re-associating with the de-lipidized lamellar protein, Ji found 36 fatty chains associating with a mole of protein at saturation. When phospholipids, galactolipids or radioactive sulfolipid were re-associated, the protein saturated at 18 molecules or 36 fatty chains per mole of protein. There seemed to be a fixed number of fatty chain association sites in a unit of membrane protein.

We should be understanding the nature and selectivity of lipid-protein interactions (Fig. 9) in mem-

branes (18). The concern of most studies seems to be either the nature and association of membrane lipids with each other or the nature and activities of the hydrophobic proteins of membranes; there has been little concern over how lipid molecules interact with membrane proteins.

Many strange contortions of the polyunsaturated fatty chains have been depicted in publications. It almost seems as if no one constructs models. To us, it is clear that the most comfortable configuration of the 22:6 fatty acid of algae or fish is not a bent system but, more reasonably, a rather straight system of helically arranged double bonds. The result is a chain equivalent in length to that of oleate or palmate. Unsaturatedness may provide the structural adaptation essential for adjusting virtual chain length in the bilayer. There even could be an interaction between the pi-binding systems of the helically arranged olefinic systems of polyunsaturated fatty acid with similar amide pi systems of the α-helical polypeptide chains of membrane proteins.

NATURE'S STARVATION INSURANCE

Hydrophobic and hydrophilic interactions play important roles in determining the internal and surface structure of lipids in biological systems. Pancreatic lipase systems hydrolyze dispersed triglycerides readily. Wax esters, on the other hand, are hydrolyzed only one-tenth as fast (19). The hydrophilic nature of the three ester linkages of triglycerides brings more of them to the surface and accessible for enzymatic attack than does the single ester linkage of wax esters (Fig. 10). It is no wonder that they are hydrolyzed, or digested only one-tenth as fast.

When Dr. Nevenzel's familiarity with wax esters and the interests in oceanic zooplankton of Drs. Paffenhofer and Lee interacted (20), it became apparent that the 70% lipid of marine copepods was actually pure liquid wax ester. These animals convert triglycerides and other lipids of oceanic microalgae to waxes and store them for use during long migrations or periods of starvation. By being so difficultly metabolized, wax ester provides energy over a longer period than could triglyceride. All midwater marine organisms, subject to uncertain food supplies, accumulate wax ester (21). It is nature's starvation insurance (22), and perhaps the earth's major nutritional energy source.

LIPIDS WITH ARSENIC AND ANTIMONY?

Yes, there are arsenolipids and stibnolipids. Perhaps life would be impossible without them. Oceanic algae, as well as all truly aquatic plants, are adapted for survival in environments severely depleted in phosphate (23). Caribbean waters and surface waters of most of the oceans are so low in phosphate that its concentration can even be lower than that of ubiquitous arsenate ($2 \cdot 10^{-8}$M) (24). Algae, then, absorb arsenate and stibnate in their quest for phosphate; they cannot discriminate between these similar or isomorphous ions. Marine products from low-nutrient surface waters, then, may contain arsenicals while products from rich upwelling seas may contain much less (25).

Phosphate and arsenate look alike; they are absorbed about equally well by algae but differ in their chemistry. Although arsenate esters are formed, they are very unstable, unlike those of phosphorous. Metabolic reductants of cells can reduce arsenate but cannot reduce phosphate. When reduced further to +1 arsenic, HAs=O, the highly toxic arsonous acid avidly esterifies free thiols of proteins (26,27). An old laboratory rat, for example, carries arsenic from its diet, on cysteine-93 of 6% of its hemoglobin molecules. Fortunately, it has little if any effect on red blood cell function. But, for the rat, it could perform a vital role in deterring invading blood parasites, perhaps the rat's secret to its environmental success.

Many algae detoxicate protein-bound arsenic by methylation and adenosylation to produce dimethylarsenoribosides and the arsenolipid. By superb NMR and IR spectrometry and X-ray crystallography Edmonds and Francesconi (28) and their collaborators (29) in Perth, Western Australia, isolated and established the structure of the algal arsenoribosides (Fig. 11).

The relationship of the arsenolipid (Fig. 12) to these water-soluble and protein-bound arsenicals was first shown by Bob Cooney (30). Again, these exotic compounds revealed themselves on radiochromatograms of algae cultured in radioarsenic or antimony (Fig. 13) media. These lipids deacylate to produce water-soluble derivatives related to other arsenic or antimony metabolites.

Most oceanic algae have only one arsenolipid, the arsenoribosyl derivative of phosphatidyl glycerol. Green algae (chlorophyta) often produce equal amounts of one or more other arsenolipids having structures much more resistant to hydrolysis (31).

What can be the function of these lipids? As part of the lipid bilayer structures (Fig. 14) of the algal outer membrane, they may well present their hydrophilic arsenoriboside moiety to the seawater environment to permit bacterial or other oxidases access to the susceptible dimethylarsenoso-group. The oceanic cacodylate [$(CH_3)_2$ AsOOH, 10^{-12} M] could thus be released (32,33). Algae such as *Dunaliella tertiolecta* produce and excrete cacodylate while brown algae, like diatoms, excrete the arsenoribosides. Brown algae of the *Sargassum* group

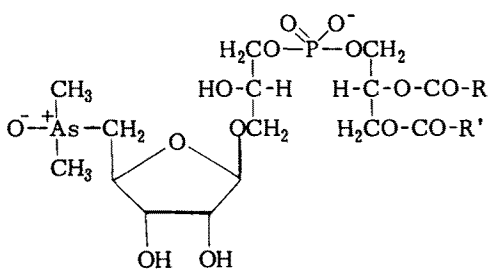
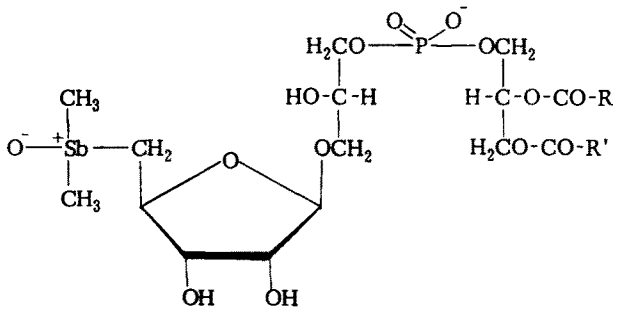


FIG. 12. The arsenolipid of aquatic plants.



The Stibnolipid of Algae

FIG. 13. The stibnolipid of marine algae, deduced from experiments analogous to those used for structure assignment of the arsenolipid (30,28).

accumulate protein-bound arsenic and appear to produce no arsenolipid.

Ancillary evidence for membrane lipid mediation in arsenic depuration comes from studies of arsenate uptake and depuration by the giant clams of the Great Barrier Reef. These animals harbor enough symbiotic algae to produce in the light more oxygen than they consume. The algae, like free-living dinoflagellate (red tide-type) algae, absorb environmental arsenate and detoxicate it, producing arsenoribosides and the arsenolipid. The clam's kidney accumulates up to 2% of arsenical compounds but is ineffective in excreting them (34,29). The clam, however, converts these arsenicals to a group of its own arsenolipids which are incorporated in the membranes of its gills, bathed in a rapid stream of fresh seawater. Although the actual arsenic content of the gills is very low, it is all in amphipathic membrane lipids and very rapidly formed. Using radioarsenate, the kinetics of gill lipid formation has been examined. It is apparent that the clam uses its gill arsenolipids as a medium for release of arsenicals to the sea. Although most of the animal's arsenolipids differ from those of algae, that is, they are separable by TLC, they appear to function similarly as carriers in arsenic depuration.

Radiochromatographic exploration has not exhausted its potential for revealing important metabolic pathways and products. With inspired questions, nature provides answers.

ACKNOWLEDGMENTS

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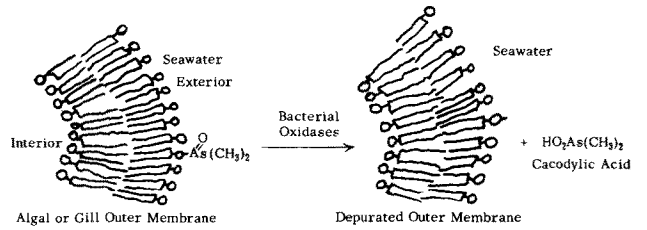

FIG. 14. Proposed mechanism for arsenic depuration mediated by membrane arsenolipids.

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