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Biochemistry

Arsoniumphospholipid in algae*

(arsenolipid/phosphatidyltrimethylarsoniumlactic acid/trimethylarsoniumlactic acid/arsenobetaine)

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ABSTRACT **A novel phospholipid containing arsenic was formed by all marine algae cultured in [^{74}As]arsenate. Components of the labeled algal extracts readily separated by two-dimensional paper radiochromatography. Base-catalyzed deacylation of the major lipid yielded a phosphodiester identical to one of the two major water-soluble compounds. Acid or enzymic hydrolysis of the phosphodiester produced a product identified as trimethylarsoniumlactic acid. The structure of the phospholipid therefore is *O*-phosphatidyltrimethylarsoniumlactic acid. Detoxication of arsenate by marine algae leads to accumulation of the arsoniumphospholipid as a major reservoir for arsenic. Its degradation to trimethylarsoniumbetaine, dimethylarsinic acid, methanearsonic acid, and arsenate in marine food chains and its metabolism in human beings are of considerable interest.**

Oceanic phosphate levels, reduced at the surface by phytoplankton and bacterial consumption, approach those of arsenate. In tropical waters, and especially the Sargasso Sea, arsenate concentrations even exceed those of phosphate (1, 2). Both ions are absorbed by their common transport system. Marine algae have developed effective procedures for detoxicating arsenate which, if it were allowed to accumulate to 1 μM, could cause serious uncoupling of phosphorylation. Bacteria (3), corals (4), and algae (5) reduce arsenate to arsenite which, similarly, would inhibit sulfhydryl enzymes of glycolysis at 1 mM unless it were further metabolized. Recognition of very low concentrations of methanearsonic acid and of dimethylarsinic acid (cacodylic acid) and their measurement by Andreae (6) and others have implicated the role of biological methylation in detoxication of arsenate in the ocean.

Algae absorb radioarsenate readily when phosphate levels are not excessive. ^{74}As-Labeled cells have been prepared and studied by many who recognized the presence of lipid arsenic components (5, 7). Recently Irgolic *et al.* (8) have reported accumulation of arsenolipid in a marine alga. In experiments designed to establish the path of arsenate in the marine food chain, we have examined the products of arsenate uptake by a variety of phytoplankton species and corals. Following methods developed in study of the path of carbon in photosynthesis (9, 10), we have resolved the products of radioarsenate reduction to reveal a simple system of substances whose biosynthesis appears to have occurred by plausible processes.

METHODS

Algal Cultures. To axenic cultures of *Chaetoceros concavicornis* and other phytoplankton species, grown to maturity in inorganic media, was added 50–100 μCi of [^{74}As]arsenate. Cells were harvested after 4 days and extracted with chloroform methanol 2:1 (vol/vol) or Bloor's solution (ether/ethanol 1:3 vol/vol).

Chromatography. Concentrated cell extracts were chromatographed two dimensionally on Whatman no. 4 paper and radioautograms were prepared (9). The orange dye, Tropaeolin 000, was an invaluable chromatographic reference.

Paper Electrophoresis. Eluted or cutout (2 × 2 mm) samples of radioactive components from paper chromatograms were separated by paper electrophoresis (10 V/cm) in a variety of buffer systems. The blue dye, indigotetrasulfonate, was used as a standard and applied as 2 × 2 mm samples onto the wet paper adjacent to each origin.

RESULTS

Thin-layer chromatography of the $CHCl_3$/MeOH extract of the marine diatom *C. concavicornis* revealed three main ^{74}As-containing spots, lipid compounds I, II, and III (Table I). Four water-soluble compounds (A, B, C, and D) were resolved by two-dimensional paper chromatography of the water extract (Table 2 and Fig. 1).

Both lipids I and II deacylated upon treatment with methanolic KOH. Chromatography revealed the identity of the deacylated compound and the naturally occurring compound B. Treatment of lipid I with phospholipase A_2 produced a compound with the same R_F as lipid II, implying that lipid II is the lyso form of lipid I.

The deacylated lipid (B) was found to acetylate in acetic anhydride/pyridine. Both mono- and diacetylated products were observed by paper chromatography. Oxidation of B with $NaIO_4$ resulted in a new compound with R_F = 0.48. A compound identical with C was produced upon treatment of the deacylated lipid with glycerophosphorylcholine diesterase. The same compound was produced upon hydrolysis in 0.1 M HCl at 100° with a half-time of 4.5 min (Fig. 2). C was also formed when the original phospholipid (I) was incubated with phospholipase D.

Compound C was found to acetylate in acetic anhydride/pyridine, indicating the presence of one free hydroxyl. C, when heated at 100° in 1 M KOH in ethanol, produced cacodylic acid. Arsenobetaine under the same conditions did not react. In addition, no reaction was detected when C was treated with methyl iodide. Compound D was identified as cacodylic acid.

Paper electrophoresis was used to determine the pK_a for each of the water-soluble radioactive and known related compounds. pH dependence of the relative mobilities (R_m) for each is reported in Table 3; the pK_a values determined from these are given in Table 4. The results show that compound C is neutral above pH 4.0 and behaves as a cation below pH 4.0. The deacylated lipid is neutral below pH 3.0 and anionic above. The intact lipid can therefore be assumed to be an anionic phospholipid at physiological pH.

Methylated arsines are highly reactive and unlikely to be observed as natural products, while tetrasubstituted arsonium ions are stable. The cationic nature of compound C at pH 3.0,

* This is paper no. 1 in a series.

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Table 1. Thin-layer chromatographic coordinates

Compound	R_F Solvent I	R_F Solvent II
Phosphatidylcholine	0.31	0.65
Lysophosphatidylcholine	0.09	0.50
Phosphatidylethanolamine	0.59	0.84
Phosphatidylinositol	0.16	0.55
I. Arsoniumphospholipid	0.23	0.55
II. Lysoarsoniumphospholipid	0.15	0.50
III. Unknown As compound	0.80	0.87

Solvent I: $CHCl_3/CH_3OH/H_2O/NH_4OH$ 130:70:8:0.5 (vol/vol). Solvent II: $CHCl_3/CH_3OH/H_2O$/HOAc 65:50:15:1 (vol/vol).

as well as its resistance to methylation, strongly indicate the presence of the trimethylarsonium moiety. Cacodylic acid is probably formed in a Hofmann-like elimination from compound C when heated in base. The initial product would probably be trimethylarsine which oxidized to cacodylic acid. Arsenobetaine, having no β hydrogen, did not give an elimination product.

The dissociation constants of B and C are indicative of a carboxylic group farther than two carbon atoms from the arsonium ion. The extremely rapid acid hydrolysis must result from cyclic interactions of the phosphate and carboxyl groups (11), facilitating cleavage of the phosphodiester. Oxidative degradation of C with either $NaIO_4$ and $KMnO_4$ or lead tetraacetate followed by aqueous bromine, reagents for selective decarboxylation and oxidation of α-hydroxyacids, yielded a product identical with arsenobetaine.

Table 2. Paper chromatographic coordinates of ^{74}As-labeled compounds in *C. concavicornis* and reference compounds*

Compound	R_F Phenol/H_2O†	R_F But/Prop/H_2O‡	^{74}As content, %
Arsenate	0.11	0.18	
Arsenite	0.26	0.45	
Methanearsonic acid	0.28	0.48	
Cacodylic acid	0.70	0.69	2
Arsenocholine	0.84	0.77	
Arsenobetaine	0.84	0.63	
A	0.60	0.23	40
Acetylated A		0.40/0.80	
B	0.66	0.17	14
Deacylated arsenolipid	0.64	0.17	
Acetylated B		0.46/0.92	
Periodate-treated B		0.48	
C	0.84	0.54	8
H^+-hydrolyzed B	0.84	0.54	
Diesterase-treated B	0.84	0.54	
Lipid treated with KOH, then H^+ at 100°	0.84	0.54	
Lipid treated with KOH, then diesterase	0.84	0.54	
Acetylated C		0.84	
D	0.71	0.70	2
Lipid	0.85	0.85	33

* Whatman no. 4 paper.
† Phenol/H_2O, 100:40 (wt/wt).
‡ Equal volumes butanol/water, 1800:121 (vol/vol), and propionic acid/water, 800:1020 (vol/vol) (9).

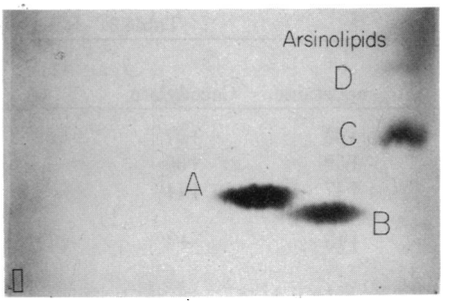
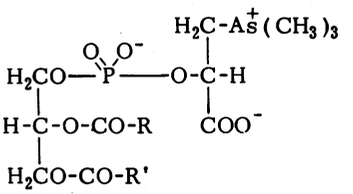


FIG. 1. ^{74}As-Labeled components of the marine diatom *C. concavicornis*. Radioautograph was prepared from two-dimensional paper chromatogram developed in the x direction with phenol/water and in the y direction with butanol/propionic acid/water solvents.

DISCUSSION

Chemical reactivities and physical properties of the ^{74}As-labeled lipid and its derivatives are consistent with the structure of *O*-phosphatidyltrimethylarsoniumlactic acid.



This is equivalent to an arsine analog of phosphatidylisoserine betaine and, like phosphatidylserine, is an acidic phospholipid. It will be interesting to explore its role in membrane structure. It seems unlikely that the presence of arsenic in the phospholipid lends significant differences in properties from those of phosphatidylcholine and phosphatidylserine. Its accumulation

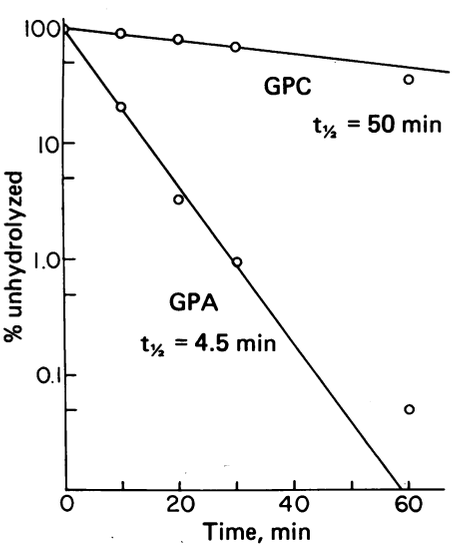


FIG. 2. Hydrolysis of glycerophosphorylcholine (GPC) and glycerophosphoryltrimethylarsoniumlactic acid (GPA) in 0.1 M HCl at 100°. Glycero[^{32}P]phosphorylcholine and [^{74}As]arsenolipid deacylation product eluted from two-dimensional paper chromatograms were hydrolyzed in closed vials. Samples withdrawn at intervals shown were chromatographed on paper with phenol/water solvent for glycerophosphorylcholine and butanol/propionic acid/water solvent for glycerophosphoryltrimethylarsoniumlactic acid. The amounts of resolved products, [^{32}P]glycerophosphate, trimethyl[^{74}As]arsoniumlactic acid, and unhydrolyzed substrates were determined by direct Geiger-Müller or liquid scintillation counting of excised radioactive areas.

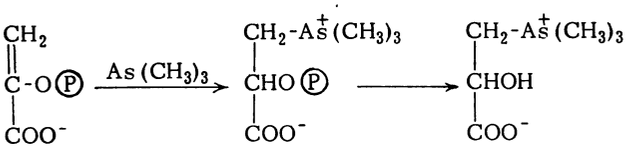
Table 3. Relative electrophoretic mobilities,* pH dependence

pH	Arsenobetaine	Cacodylate	A	B	C	C (KOH, 100°)	D	Deacylated lipid (H^+ hydrolysis)
1.2	+60	+57					+57	
1.4	+59	+36				+36	+36	
1.8	+47	+14				+13		
2.0			0	0	+33			+33
2.5	+15	+4	0	0	+33			+33
3.0			−10		+23			
3.2			−13	−10	+18			+20
3.4			−17	−12	+15			+18
3.5	+1	0	−21	−14	+9	0	0	
3.75			−26	−19	+6			+6
4.2			−28	−23	+2			+2
4.5			−31		0			0
5.0			−31	−26	0			0
6.0		−28					−30	
7.0		−40				−42	−44	
8.0			−32	−29	0			0

+, Cation; −, anion.
* R_m compared to indigotetrasulfonate × 100.

in marine algae and animals and its probable function as a detoxicated form of arsenic appears related to its structural similarities rather than differences.

The structure of trimethylarsoniumlactic acid suggests its probable biosynthesis from phosphoenolpyruvate by nucleophilic reaction with trimethylarsine.



Both oxidative chemical degradation reported here and apparent biological degradation of the arsenophospholipid yield trimethylarsoniumbetaine, the structure of which has recently been elucidated (12). This stable compound must occupy a central role in the ultimate biological degradation of lipid arsenic to oceanic arsenate. Ubiquitous methanearsonate and dimethylarsinate in the ocean (6) appear related to Hofmann elimination products of this compound as well as biomethylation processes (13).

Occurrence and transformations of arsenic in the marine environment have been ably reviewed by Lunde (14). Lipids from mussels, shrimps, and snails (15) contained arsenolipids (16) which readily passed through the food chain. Of all the extensive efforts of many laboratories to identify intermediates, none appears related to those reported here. As in the case of identification of other plant lipids, the power of the radiochromatographic method offered an unequivocal and comprehensive approach.

Table 4. Dissociation constants determined from electrophoreses

Compound	pK_a observed	pK_a reported
A	3.3	
B	3.5	
C	3.4	
D	1.6, 6.0	
Cacodylic acid	1.6, 6.0	1.57, 6.2
Arsenobetaine	2.1	

Note Added in Proof. Structure elucidation reported in this paper has been verified by exact cochromatography of the ^{74}As-labeled natural substance with synthetic trimethylarsoniumlactate in several paper chromatographic solvent systems and under critical pH electrophoresis. The product was synthesized by reaction of trimethylarsine with glycidic acid kindly provided by Meinrat Andreae and Israel Zelitch, respectively. Formation of the isomeric serine betaine analog and its reactivity will be reported elsewhere. The assistance of George Jolliffe, Jr., is deeply appreciated.

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