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Phosphonic Acid Metabolism in *Tetrahymena**

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ABSTRACT: The uptake and metabolism of 2-[^{14}C]-(β-phosphono)alanine and 1,2-[^{14}C]aminoethylphosphonic acid by *Tetrahymena pyriformis* were studied *in vivo*. The aminoethylphosphonic acid was taken up and incorporated into phospholipids to the extent of 10% without any degradation. About 19% of the (β-phosphono)alanine taken up by the cell during growth was found as aminoethylphosphonic acid in the phospholipids.

No (β-phosphono)alanine was detected at the phospholipid level. Some of the radioactive label from (β-phosphono)alanine was also found in the fatty acids and glycerol ethers.

In the past few years the phospholipids of *Tetrahymena pyriformis* have been the center of intensive interest because of the presence of an unusual phospholipid base, 2-aminoethylphosphonic acid (Kandatsu and Horiguchi, 1962). In the phospholipids AEP[1] occurs linked to ceramide (Carter and Gaver, 1967) and to diglyceride and 2-acylchimyl alcohol (Rosenberg, 1964; Thompson, 1967). AEP is also present in the free form and covalently linked to an insoluble residue (Rosenberg, 1964).

In addition to AEP, another phosphonic acid, (β-phosphono)alanine, is found in *Tetrahymena* (Kittredge and Hughes, 1964). While absent from the lipids of *Tetrahymena*, it is present in the free form and in the insoluble residue. PAla is presumed to be a biosynthetic precursor of AEP (Kittredge and Roberts, 1969). The literature on biosynthesis of the carbon–phosphorus bond has been recently reviewed (Kittredge and Roberts, 1969).

Liang and Rosenberg (1966) showed that homogenates of *Tetrahymena* could incorporate ^{32}P-labeled AEP into phospholipids. Warren (1968) reported a small conversion (0.04%) of ^{32}P-labeled PAla to AEP by extracts of *Tetrahymena*. While these authors have reported the ability of *Tetrahymena* to perform these reactions, there has been no report of the actual capacity of the organism to metabolize AEP and PAla, *in vivo*.

In the work presented here we have studied the uptake of 1,2-[^{14}C]AEP and 2[^{14}C]PAla by *Tetrahymena* during its growth and their metabolism by the organism.

Materials

2-[^{14}C]Sodium pyruvate (10 μCi/μmole) was obtained from International Chemical and Nuclear Corp., and 1,2-[^{14}C]-ethanolamine-HCl (117 μCi/μmole) from Tracerlab. Pyruvic acid was purchased from Calbiochem and ethanolamine from Aldrich Chemical Co.

Dimyristoylglyceryl-AEP (PnE) was the gift of Dr. Erich Baer.

The sources of other materials have been previously reported (Smith and Law, 1970).

Methods

Synthesis of 2-[^{14}C]Phosphonoalanine. 2-[^{14}C]Sodium pyruvate (500 μCi) to which 750 μmoles of nonradioactive pyruvic acid had been added was used for the synthesis of 2-[^{14}C]-PAla. The intermediate *N*-acetyldehydroalanine was prepared by the method of Wieland *et al.* (1957), and PAla by the procedure of Chambers and Isbell (1964). To pyruvate was added 73.4 mg of acetamide and 5 ml of benzene. The mixture was heated under reflux for 5 hr with a trap containing $CaSO_4$ to remove the water formed in the reaction. The benzene was then evaporated under nitrogen and a fivefold molar excess of each of trimethyl phosphite and dimethyl phosphite was added. After heating on a steam bath for 90 min, the reaction mixture was left at room temperature. After

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[1] Abbreviations used are: AEP, 2-aminoethylphosphonic acid; PAla, (β-phosphono)alanine; PnE, diacylglyceryl-AEP; PE, phosphatidylethanolamine.

36 hr 5 ml of concentrated HCl was added and the mixture was hydrolyzed for 55 hr under reflux. Following hydrolysis the mixture was decolorized with activated charcoal, concentrated, and applied to a column of Dowex 50 H^+-X8 (4 × 30 cm) ion-exchange resin. The column was eluted with water and 10-ml fractions were collected. The fractions were monitored for radioactivity by removing aliquots for scintillation counting. The tubes in each peak of radioactivity were pooled and aliquots were spotted for paper chromatography in phenol–H_2O, 4:1 (chromatographic chamber saturated with HOAc vapor). The fraction containing PAla was identified by comparison of the radioactive material with PAla standard (R_F 0.5). The fraction was concentrated and PAla was precipitated by addition of a large excess of EtOH. The residue was dissolved in water and EtOH was again added. The final product was radiochemically pure as judged by paper chromatography in phenol–H_2O, 4:1, and BuOH–HOAc–H_2O, 5:3:1. The final yield was 10% by radioactivity with a specific activity of 0.6 μCi/μmole based on the starting material.

Synthesis of 1,2-[^{14}C]Aminoethylphosphonic Acid. 1,2-[^{14}C]AEP was synthesized by the method of Kosolapoff (1947); the intermediate bromoethylphthalimide was prepared by the method of Soine and Buchdahl (1962). 1,2-[^{14}C]Ethanolamine-HCl (500 μCi, 117 μCi/μmole) to which 2.89 mg of unlabeled ethanolamine (total 51 μmoles) had been added was mixed with 9.9 mg of phthalic anhydride (67 μmoles), and the mixture was heated on a steam bath for 1 hr. The mixture was cooled, 1 drop of PBr_3 was added, and the mixture was again heated on the steam bath. After 1 hr the reaction was stopped by cooling and the mixture was evacuated with a water aspirator to remove excess PBr_3.

To the crude bromoethylphthalimide was added an equimolar amount of triethyl phosphite and the mixture was refluxed for 4.5 hr at 160°. HBr (3 ml, 48%) was added and the mixture was again refluxed for 16 hr. The hydrolysate was decolorized with activated charcoal and evaporated to dryness. Water was added to the residue and the mixture was evaporated several times to remove all the HBr. The crude material was applied to a column of Dowex 50H^+-X8 (4 × 30 cm). The column was eluted consecutively with 500 ml of water, 500 ml of 20% NH_4OH, and 1000 ml of concentrated NH_4OH. Fractions (10 ml) were collected and the radioactivity was monitored as described for the synthesis of PAla. The phenol–H_2O chromatographic system was used to locate the AEP (R_F 0.8) and determine its radiopurity. The AEP was precipitated twice from EtOH to remove traces of contamination. The final yield was 5% (10 μCi/μmole).

Culturing. Tetrahymena pyriformis WH14 was grown on a proteose-peptone medium containing either [^{14}C]PAla or [^{14}C]AEP and harvested, and the cell fractions (lipid-soluble, aqueous, and residue) were isolated as previously described (Smith and Law, 1970). The radioactive compounds were present during the complete growth of the organism which was carried out for 24 hr (log phase) or 48 hr (stationary phase).

Chromatography. Thin-layer chromatography on silica gel G was carried out using the solvent systems of Thompson (1969), $CHCl_3$–HOAc–MeOH–H_2O, 75:25:5:1.8, and Hori *et al.* (1969), $CHCl_3$–MeOH–HOAc–H_2O, 100:20:12:5, for phospholipids and hexane–Et_2O–HOAc, 50:50:1, for neutral lipids. Acid hydrolysis of the phospholipids was carried out for 5 hr in 6 N HCl in the autoclave (Scarborough and Nyc, 1967). The hydrolysate was extracted four times with 4 ml of $CHCl_3$, and the aqueous portion was dried and neutralized with 1,2-butylene oxide in EtOH before spotting on Whatman No. 1 paper for chromatography in phenol–H_2O, 4:1 (over HOAc). Phospholipase A hydrolysis was carried out by the method of Hildebrand and Law (1964). At the end of the hydrolysis the solvent was evaporated under nitrogen, the residue was suspended in 4 ml of 0.1 N HCl, and the solution was extracted with four 4-ml portions of $CHCl_3$. The material soluble in $CHCl_3$ was concentrated and applied to plates for thin-layer chromatography. Alkaline hydrolysis of the phospholipids was carried out by the method of Hori *et al.* (1967). After a 24-hr incubation at 37°, the mixture was dried, 4 ml of 0.1 N HCl was added, and the resulting solution was extracted with $CHCl_3$.

Gas chromatography of fatty acid methyl esters was performed on a 4-ft preparative column of 10% EGSS-X on Gas-Chrom Q in a Hewlett-Packard gas chromatograph, Model 402, equipped with a splitter. The effluent from the splitter was collected in glass tubes at 10-min intervals and washed into scintillation vials with scintillation fluid for determination of radioactivity in a Packard Tri-Carb scintillation counter, Model 3003.

TABLE I: Distribution of Radioactive Label in the Various Fractions of *Tetrahymena* Grown on 2-[^{14}C]PAla.

Fraction	Stationary Phase (48 hr), Radioactivity		Log Phase (24 hr), Radioactivity	
	cpm × 10^{-3}	%	cpm × 10^{-3}	%
Medium + KCl wash	29,662	97.74	20,566	99.10
Lipids	216	0.64	38	0.15
Aqueous	477	1.57	147	0.70
Residue	12	0.03	5	0.02

Results

Growth of Tetrahymena on 2-[^{14}C]Phosphonoalanine. Cells were grown on a medium containing 3 × 10^7 cpm of 2-[^{14}C]-PAla for 24 or 48 hr. The cells were harvested and the various cell fractions were isolated according to the procedure of Smith and Law (1970). The results are presented in Table I.

The total uptake of radioactive compound for 48-hr cells was 2.24% of the material present in the medium of which 0.64% was present in the lipids. The lipids were further fractionated on a column of Unisil into neutral lipid, phospholipid, and polar lipid fractions (Table II).

Thin-layer chromatography of the neutral lipid fraction in hexane–Et_2O–HOAc 50:50:1 revealed that the radioactive material was associated predominantly with the triglyceride fraction and not with contaminating phospholipids, all of which remained at the origin in this solvent system.

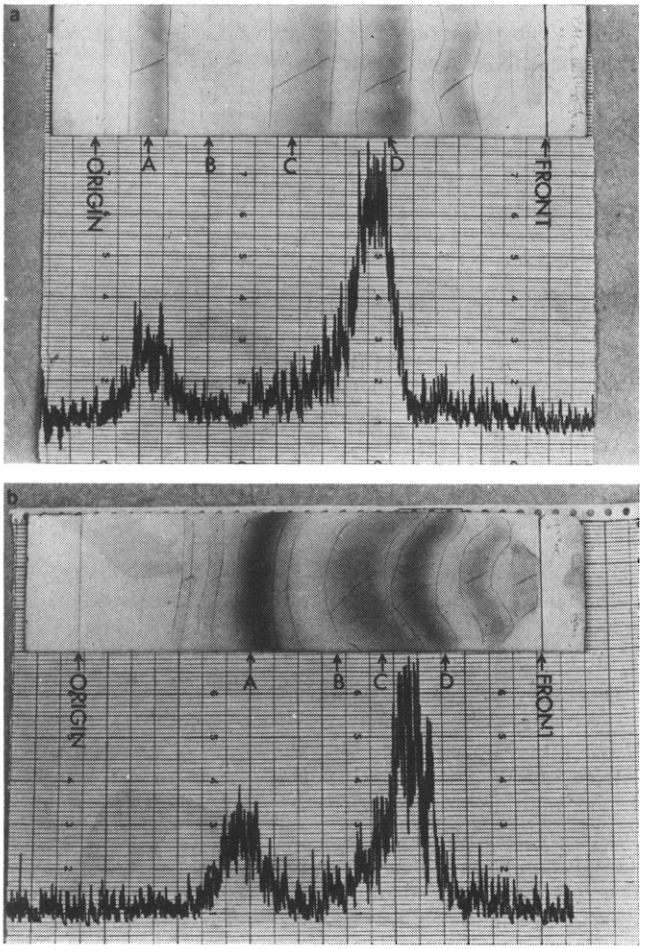

FIGURE 1: Thin-layer chromatograms of the phospholipids from *T. pyriformis* grown on 2-[^{14}C]PAla for 48 hr: (a) chromatographic system of Thompson (1969); (A) lecithin, lyso-PE, lyso-PnE; (B) phosphatidylserine; (C) PE; (D) PnE; (b) chromatographic system of Hori *et al.* (1969); (A) lecithin, lyso-PE, lyso-PnE; (B) ceramide AEP (no standard available, R_F indicated by Hori *et al.*, 1969); (C) PE; (D) PnE.

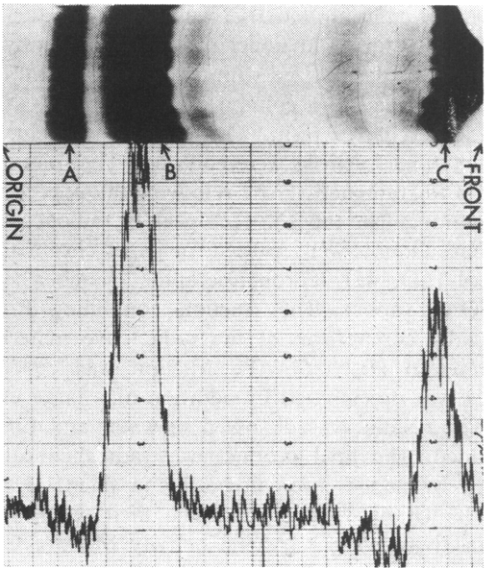

FIGURE 2: Thin-layer chromatogram (Hori system) of the $CHCl_3$-soluble material after phospholipase A hydrolysis of the phospholipids from PAla grown cells: (A) lysolecithin; (B) lyso-PE, lyso-PnE; (C) fatty acids.

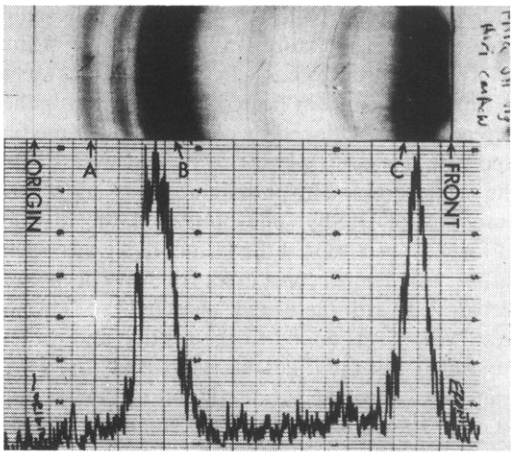

FIGURE 3: Thin-layer chromatogram (Hori system) of the $CHCl_3$-soluble material after alkaline hydrolysis of the phospholipids from PAla grown cells: (A) lysolecithin; (B) lyso-PE, lyso-PnE; (C) fatty acids.

Thin-layer chromatography of the phospholipids in the systems of Thompson or Hori revealed that most of the radioactivity was associated with the PnE fraction (both systems cleanly separate PnE from AEP) with minor amounts associated with other phospholipids (Figure 1a,b).

Phospholipase A hydrolysis of the phospholipids and thin-layer chromatography of the $CHCl_3$-soluble material (Figure 2) revealed that in addition to the lyso-PnE formed, some of the radioactivity was also associated with the fatty acids. Strong alkaline hydrolysis of the phospholipids and thin-layer chromatography of the $CHCl_3$-soluble material gave much the same pattern as the phospholipase A hydrolysis (Figure 3). About 40% of the radioactivity remained $CHCl_3$ soluble after alkaline hydrolysis. The material in the lyso-PnE area of the chromatogramrepresented the glyceryl ether portion of the phospholipids. Again, labeled fatty acids were present.

To confirm the fatty acid nature of this material, the silica gel was scraped from the thin-layer plate and the fatty acids were eluted with MeOH. The methyl esters were formed by treatment with diazomethane generated from *N*-methyl-*N*-nitrosourea and the fatty acid methyl esters were examined by gas chromatography. The radioactivity was evenly distributed in the fatty acids in proportion to their abundance in *Tetrahymena* (Erwin and Bloch, 1963), although as shown in Figure 1, most of the labeled fatty acids appeared to be associated with the PnE. The recovery of fatty acid esters from the gas chromatographic column was only 30% as compared with 60% recovery of standard esters. A large portion of the radioactive compounds thus remains uncharacterized.

Acid hydrolysis of the phospholipids and paper chromatography of the resulting water-soluble material indicated that all of the water-soluble radioactivity was associated with

TABLE II: Distribution of Radioactivity in the Various Lipid Fractions from PAla-Grown Cells.[a]

Eluting Solvent	Fraction	48-hr Cells			24-hr Cells		
			Radioactivity			Radioactivity	
		Wt, mg	cpm × 10^{-3}	%	Wt, mg	cpm × 10^{-3}	%
$CHCl_3$	Neutral lipid	25.2	21	9.8	13.5	3.0	8.0
MeOH	Phospholipid	193.3	194	89.9	29.8	34.6	91.2
10% HOAc in MeOH	Polar lipid	15.7	1	0.4	5.1	0.3	0.8

[a] The lipids were fractionated on a column of Unisil (15 g). The neutral lipid, phospholipid, and polar lipid fractions were eluted with $CHCl_3$, MeOH, and 10% HOAc in MeOH, respectively.

TABLE III: Distribution of Radioactivity in the Various Portions of the Phospholipid Molecule.[a]

	%	Radioactivity, cpm (%)		
		Diacylphospholipids		Glyceryl Ether Phospholipids
Position 1 (α')	14.5	1860 (7.2)		1852 (7.3)
Position 2 (β) (fatty acids)	18.4		4698 (18.4)	
Position 3 (α) (base-AEP)	67.0	8190 (32.1)		8900 (34.9)

[a] The phospholipids were treated with phospholipase A for 24 hr and the $CHCl_3$-soluble material was extracted. (All of the radioactivity was found in the $CHCl_3$.) The fatty acids and lysophosphatides were separated on a column of Unisil by elution with $CHCl_3$ (fatty acids) and MeOH (lysophosphatides). The lysophosphatides were then treated with base for 24 hr and the resulting $CHCl_3$-soluble material separated on Unisil as before. The resulting lysoglyceryl ether phosphatides were hydrolyzed in acid to release the bases.

AEP (Figure 4). The paper strip was cut into 2-cm pieces, placed in scintillation vials, and counted to look for small amounts of radioactivity below the detection limits of the strip counter (about 5%). No PAla or any other radioactive material could be detected. About 70% of the radioactivity in the phospholipids was associated with AEP.

In a further experiment a portion of the phospholipids was treated with phospholipase A, followed by base and then by acid, with separation of the $CHCl_3$-soluble material on a column of Unisil to determine the relative amount of radioactivity in the glyceryl ether and diacylphospholipids (Table III).

The value of 70% of the radioactivity in the lipids present as AEP represents about 19% of the total uptake of the radioactive PAla. AEP could not be detected in the aqueous portion of the cell extract, although at least two other major components in addition to PAla were present. Not enough radioactive material was present in the residue to identify any particular compound after the material had been hydrolyzed.

In an attempt to reduce the amount of breakdown of PAla relative to AEP formed, cells were grown on [^{14}C]PAla for only 24 hr (log phase). Only 0.8% of the material was taken up with 0.15% present in the lipids as compared with 2.24% and 0.64% for the cells isolated in the stationary phase (Table I). The lipids were further separated into the neutral lipid, phospholipid, and "polar lipid" classes (Table II).

Thin-layer chromatography of the phospholipids revealed the same pattern as found with the phospholipids from stationary phase cells. Phospholipase A, base, and acid hydrolysis of the phospholipids demonstrated that again, about 70% of the radioactivity was associated with AEP and the remainder with the fatty acids and glyceryl ethers. No significant difference in the distribution of radioactivity in the log phase cells from that in stationary phase cells was found.

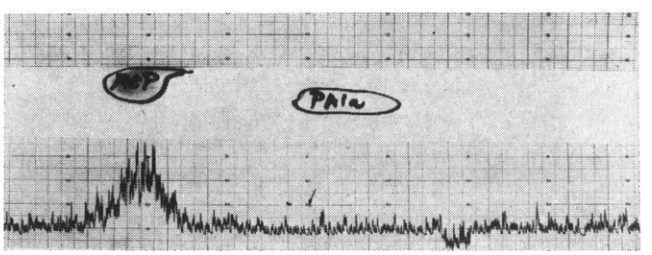

FIGURE 4: Paper chromatogram (phenol–H_2O, 4:1) of the water-soluble material from the acid hydrolysate of PAla-grown cells.

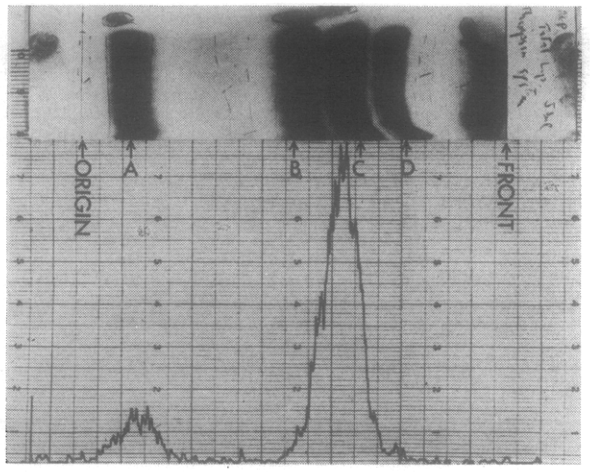

FIGURE 5: Thin-layer chromatogram (Thompson system) of the phospholipids from AEP-grown cells: (A) lecithin, lyso-PE, lyso-PnE; (B) PE; (C) PnE; (D) cardiolipin.

Growth of Tetrahymena on 1,2-[^{14}C]Aminoethylphosphonic Acid. Cells were grown on a medium containing 9×10^6 cpm of 1,2-[^{14}C]AEP for 48 hr. The distribution of radioactive label in the various cell fractions is given in Table IV. The lipids were further fractionated on a column of Unisil (Table V).

Essentially all of the radioactivity was associated with the phospholipid fraction. Thin-layer chromatography revealed that most of the radioactive material was present as PnE with a small amount as lyso-PnE (Figure 5). No radioactive compound with an R_F value (Hori *et al.*, 1969) of ceramide AEP was observed. Acid hydrolysis demonstrated that all of the radioactivity was found in AEP. Phospholipase A and alkaline hydrolysis of the phospholipids confirmed that much of the radioactive phosphonic acid base was bound to a glycerol ether (Figures 6 and 7).

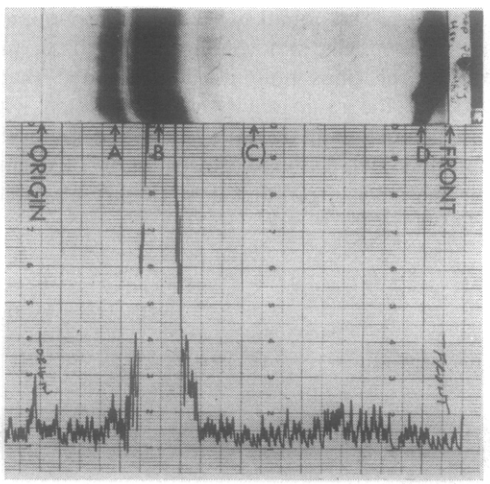

FIGURE 6: Thin-layer chromatogram (Hori system) of the $CHCl_3$-soluble material after phospholipase A hydrolysis of the phospholipids from AEP-grown cells: (A) lysolecithin; (B) lyso-PE, lyso-PnE; (C) ceramide AEP (no standard available, R_F indicated by Hori *et al.*, 1969); (D) fatty acids.

TABLE IV: Distribution of Radioactive Label in the Various Cell Fractions of *Tetrahymena* Grown on [^{14}C]AEP.[a]

Fraction	cpm $\times 10^{-3}$	%
Medium + KCl wash	8,135	90.4
Lipids	817	9.1
Aqueous	42	0.5
Residue	9	0.1

[a] Cells were grown on a medium containing 9×10^6 cpm of 1,2-[^{14}C]AEP for 48 hr at 37°. The cells were harvested and the various fractions were isolated as previously described.

About one-third of the radioactive material became water-soluble after alkaline hydrolysis. Examination of the aqueous and residue portion of the cells revealed that there was no detectable degradation of the AEP.

Discussion

The experiments presented in this paper have provided evidence for the uptake of AEP and its incorporation into PnE and for the conversion of PAla into AEP. While these experiments do not indicate whether this conversion occurs at the phospholipid level or in the free form, several lines of evidence suggest that the latter might be the case. No labeled PAla was found in phospholipids even though diglyceride-PAla has been suggested as an intermediate in PnE biosynthesis. Indeed, Kittredge and Hughes (1964) were unable to demonstrate any PAla at the phospholipid level in their original isolation of the compound from *Tetrahymena*.

Liang and Rosenberg (1966) have shown that, *in vitro*, chemically synthesized CMP-AEP can transfer its AEP moiety to a diglyceride to form PnE. They also showed that, *in vitro*, AEP could be found in a form which was absorbed to charcoal following incubation with CTP. Furthermore, no pathway is known for the incorporation of serine phosphate, of which PAla may be considered an analog, into phospholipids. Serine is incorporated

TABLE V: Distribution of Radioactivity in the Various Lipid Classes from AEP-Grown Cells.[a]

Elution Solvent	Fraction	cpm $\times 10^{-3}$	%
$CHCl_3$	Neutral lipids	0.7	0.1
MeOH	Phospholipids	774.4	99.1
10% HOAc in MeOH	Polar lipids	6.3	0.8

[a] The lipids from AEP-grown cells were separated into neutral lipids, phospholipid, and polar lipid classes by elution from a column of Unisil silicic acid (15 g) with $CHCl_3$, MeOH, and 10% HOAc in MeOH, respectively.

into phospholipid either by exchange of free serine with PE (Borkenhagen *et al.*, 1961) or by reaction with CDP-diglyceride (Kanfer and Kennedy, 1964). Alternatively it is tempting to postulate that conversion takes place at the phospholipid level by analogy with the conversion of serine into ethanolamine, but, since no labeled PAla was found at the phospholipid level, then decarboxylation must be quite efficient if PAla is to be considered a precursor of the diglyceride ester of AEP.

In spite of the breakdown of PAla and recycling of the carbons, the 19% conversion of PAla into AEP probably does represent a direct conversion. 6-[^{14}C]Glucose, the best nonphosphonic acid precursor of AEP previously discovered (Trebst and Geike, 1967), is incorporated to the extent of only 0.1% of material taken up by the cells. Pyruvate and acetate are converted into AEP in less than 0.1% yields.

In addition, it should be kept in mind that the synthetic PAla is a racemic mixture while the natural PAla is probably a single isomer. Thus the 19% conversion may well represent 38% of a single isomer. Moreover, there may have been unequal uptake of the two isomers. Indeed there may well be preferential breakdown of the unnatural isomer.

The redistribution of carbon from labeled PAla has some curious features. It is unlikely that the conversion of PAla to AEP can be explained by degradation to acetate or pyruvate and incorporation of these into product, for acetate and pyruvate are poor precursors of AEP as noted above. Conversion of the PAla into acetate is very likely, because the fatty acid and glyceryl ether groups of the phosphonolipid become labeled. The puzzling fact is that fatty acid chains of other cellular lipids, *e.g.*, phosphatidylethanolamine, contained very much less label. One possible explanation of this anomaly would be that phosphonic acid metabolism takes place in a specialized compartment within the cell and that there is not general exchange with cellular pools during biosynthesis of phosphonolipids. When phosphonic acids are supplied from outside the cell, they may be transported to this special location for both incorporation into lipids and for degradation in the case of PAla.

Although *Tetrahymena* is reported to contain ceramide-AEP to the extent of 15% of the total phospholipids (Carter and Gaver, 1967), we were unable to detect any incorporation of AEP into this phospholipid. Possibly the strain of *Tetrahymena* used in these experiments contains very little if any ceramide-AEP as opposed to the strain in which this compound was detected.

LaNauze and Rosenberg (1968) have shown that extracts of *Bacillus cereus* can convert AEP into phosphonoacetaldehyde and then into acetaldehyde and inorganic phosphate. Also, Roberts *et al.* (1968) have shown that cell-free preparations of *Tetrahymena* catalyze the breakdown of AEP and also of PAla. The lack of *in vivo* degradation of AEP in our experiments suggests the absence of any general mechanism for the enzymatic cleavage of the C–P bond in *Tetrahymena*.

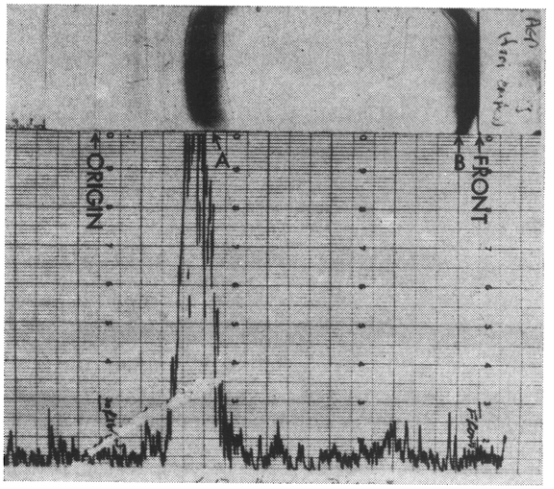

FIGURE 7: Thin-layer chromatogram (Hori system) of the $CHCl_3$-soluble material after alkaline hydrolysis of the phospholipids from AEP-grown cells: (A) lyso-PE, lyso-PnE, (B) fatty acids.

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