Intracellular Distribution of Ciliatine (2-Aminoethylphosphonic Acid) in *Tetrahymena*

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The mitochondria of *Tetrahymena pyriformis* (GL) contains approximately 19 percent of ciliatine, highest concentration found in total cell; other fractions contain smaller amounts of ciliatine. In labelling experiments, the mitochondria fraction showed higher value than that found in other fractions. This result indicates that the rate of biosynthesis of ciliatine is predominant in the crude mitochondria. In contrast, incorporation of ^{32}P -phosphorus into phosphonate was not found in the microsome fraction.

While the ester linkage is the most common mode of bonding phosphorus to carbon in nature, phosphonic acids, which contain direct carbon-to-phosphorus bonds, have been found in a variety of organisms.¹⁾ Compounds containing these bonds are generally structural analogs of naturally occurring phosphate esters. One of the most widely distributed phosphonic acid is 2-aminoethylphosphonic acid (AEP, Ciliatine), an analog of phosphorylethanolamine. AEP occurs in the bound form to proteins and lipids as well as in the free form and sometimes accounts for as much as one-half of the total phosphorus of the organism.¹⁾ The carbon-phosphorus linkage is highly resistant to hydrolytic enzymes²⁾ and, in addition, the presence of AEP in phospholipids prevents their degradation by certain phospholipases.³⁾ A possible physiological role for such lipids, termed phosphonolipids, has been suggested by Kittredge and Roberts¹⁾

and by La Nauze.⁴⁾

Recently, Smith *et al.*⁵⁾ reported that about 78% of phosphorus of lipids fraction of *Tetrahymena* cilia was of phosphonate.

This led us to examine the cellular distribution of ciliatine in *Tetrahymena pyriformis*.

MATERIALS AND METHODS

Cells of *Tetrahymena pyriformis* (DL), were grown at 26°C for 67 hr in a supplemented proteose-peptone (2%), yeast extract (0.1%) and dextrose (0.5%) medium. Then, the addition of 3 mCi of ^{32}P -ortho-phosphate was achieved by injecting the substrate (dissolved in 1 ml of distilled water) below the surface of the rapidly stirred medium. After 20 min, cells were harvested by chilling the medium to 4°C in a low temperature room and centrifuged for 5 min at 600 $\times$ g to remove whole cells. The organisms (2.5 ml) were then resuspended in 8 ml of mixed solution of sucrose (0.2 M) and NaCl (0.18%) and dropped into 39 ml of mixed solution (pH 8.3, 0°C) of glycerol (70%) - tris (20 mM) - KCl (50 mM) - MgSO₄ (2.5 mM) - thio-glycolic acid at -21°C in NaCl-ice bath.

This cell suspension was homogenated in a Waring

1) J. S. Kittredge and E. Roberts, *Science*, **164**, 37 (1967).

2) E. Roberts, D. S. Simonsen, M. Horiguchi and J. S. Kittredge, *Science*, **159**, 886 (1968).

3) G. A. Thompson, Jr., *Biochim. Biophys. Acta*, **176**, 330 (1969).

4) J. La Nauze, Thesis, John Curtin School of Medical Research, Canberra, Australia (1969).

5) J. D. Smith, W. R. Snyder and J. H. Law, *Biochem. Biophys. Res. Comm.*, **39**, 1163 (1970).

blender (Marusan Homo Blender No. 13950, 36-1, Sakuma Seisakusho Ltd.) (speed 1, 90 v) for 20 sec and centrifuged at $5,500 \times g$ for 10 min. Cilia were then collected from supernatant by centrifugation at $100,000 \times g$ for 90 min (cilia 1).

Deciliated cell precipitate was disrupted in a Waring blender (60 v) for 30 sec, in glycerol buffer, containing 0.2 M sucrose and 0.18% NaCl. Homogenates were centrifuged at $5,500 \times g$ for 10 min. The resulting supernatant was centrifuged at $100,000 \times g$ for 90 min to obtain a cilia 2. Deciliated cell precipitate was disrupted in a Potter—Elvehjem Homogenizer at $600 \times g$ for 15 min, in 40 ml of mixed solution of 0.25 M sucrose and 3 mM CaCl_2 . Homogenate was centrifuged at $600 \times g$ for 15 min. The resulting supernatant was mixed with 0.55 M sucrose and centrifuged at $6,500 \times g$ for 15 min to obtain crude mitochondria (precipitate). The resulting supernatant was centrifuged at $105,000 \times g$ for 90 min to obtain a microsomal fraction (precipitate) and soluble fraction (supernatant).

In labeling experiments, radioactivity in fractions was assayed with a Aloka Model JDC-103 Scintillation Spectrometer. Phosphonate- ^{32}P was determined by the

method described in Fig. 1. Inorganic phosphate was estimated by the method of Dryer *et al.*⁶⁾ The organic phosphates were measured as inorganic phosphate after hydrolysis in 50 volumes of 6 N HCl at 110°C for 48 hr; under these conditions, the above organophosphonic acids were resistant to hydrolysis. Total phosphorus was determined as inorganic phosphate after ashing of the samples with 6 N H_2SO_4 for about 3 hr and with three drops of perchloric acid for an hour.

Phosphorus in organophosphonic acid was determined as the difference between the total and the acid-labile phosphate. Ester phosphorus was determined by the method of Aalbers and Bieber.⁷⁾ For the separation of phosphonate thin-layer chromatography on Silica gel C was carried out using a solvent system, *n*-butanol:acetic acid:water, 4:1:2, and spots were developed with ninhydrine and Rosenberg Reagent.⁸⁾

RESULTS

In repeated trials, 13 percent of the phosphorus in whole cells was found as phosphonates (Table I). The amount of phosphonate phosphorus in isolated mitochondria and microsomes was 19 and 4 percent and that of cilia and soluble fraction was not more than

FIG. 1. Method for Determination of Phosphonate- ^{32}P in Hydrolysate of *Tetrahymena pyriformis*.

TABLE I. CELLULAR DISTRIBUTION OF PHOSPHONATE IN *Tetrahymena pyriformis*

Fraction	$\frac{\text{C}-^{32}\text{P}}{\text{T}-^{32}\text{P}} \times 100$	T-P (mg)	C-P (mg)	$\frac{\text{C}-\text{P}}{\text{T}-\text{P}} \times 100$
Whole cells	0.39	76.3	10.1	13.2
Cilia (1+2)	0.41	28.2	0.3	1.1
Crude mitochondria	0.75	7.8	1.5	19.2
Crude microsomes	—	12.8	0.5	4.2
Soluble fraction	0.58	18.5	0.3	1.5

Cells were incubated for 20 min with ^{32}P -ortho-phosphate (3 mCi).

C-P: Phosphonate-p, T-P: Total-P

6) R. L. Dryer, A. R. Tammes and J. I. Routh, *J. Biol. Chem.*, **225**, 177 (1957).

7) J. A. Aalbers and L. L. Bieber, *Anal. Biochem.*, **24**, 443 (1968).

8) H. Rosenberg, *J. Chromatography*, **2**, 487 (1959).

2 percent, respectively. The phosphonate content of the mitochondria is at least five-fold that of microsomes.

In labeling experiments, cells were incubated for 20 min with ^{32}P -orthophosphate.

In whole cells, 0.4 percent of the phosphorus- ^{32}P was found as phosphonates- ^{32}P . The percentage of radioactivity of phosphonate phosphorus in isolated cilia, mitochondria and soluble fraction was 0.4, 0.8 and 0.6 percent, respectively. But, in isolated microsomes was not detected as ^{32}P -phosphorus-phosphonates.

Thin-layer chromatography of the strong acid hydrolysate (6N, HCl, 24 hr, 120°C) of mitochondria fraction indicated two Rosenberg reagent-positive compounds to be present (see Fig. 2).

One of these two compounds had the same R_f value (0.22) as that of ciliatine in the *n*-butanol solvent system used. Moreover, chromatography of a mixture of the hydrolysate and AEP did not result in any resolution be-

tween the authentic AEP and the compounds in the hydrolysate. Because of the small quantity of material available, no attempt was made for isolation; AEP was a minor component of the hydrolysate.

DISCUSSION

About 19 percent of the phosphorus in mitochondria was found as phosphonate (Table I). And also, in the case of mitochondria, it was identified as AEP.

Kennedy and Thompson⁹⁾ found that approximately 32 percent of the phospholipids from the mitochondria of *Tetrahymena pyriformis* contained ciliatine (AEP).

Isolated pellicles are likely to contain slightly higher amounts of AEP—up to 40 percent (Details of pellicle isolation to be reported elsewhere). The relative amounts of ciliatine in cilia were much lower than in any other part of the cell examined by us. Kennedy and Thompson¹⁰⁾ have shown that 50 to 75 percent of the phosphorus in ciliary lipids was found as phosphonates.

Smith, Snyder and Law⁵⁾ found that lipid fraction extracted from isolated cilia of *Tetrahymena pyriformis* contained a high proportion of phosphonolipids and much smaller amounts of phospholipids, the major component being an alkoxy acyl glycerol ester of ciliatine (AEP), and corresponding deacylated derivative. Carter and Gaver¹⁰⁾ reported that about 12% of the polar lipid fraction of *Tetrahymena* was long chain base.

Culbertson¹¹⁾ reported that the lipids of *Tetrahymena* cilia were similar to those from whole cells. *Tetrahymena* is the organism reported to show a significant enrichment of phosphonates in its outer membranes. The

FIG. 2. Thin-layer Chromatogram of Mitochondrial Hydrolysate.

Thin-layer chromatographic plate developed with a mixture of *n*-butanol : acetic acid : water: 4:1:2 and spots were made visible by spraying with ninhydrine and Rosenberg reagent.

Lane 1, ciliatine (synthetic); lane 2, P_i ; lane 3, mitochondrial hydrolysate; lane 4, ciliatine + mitochondrial hydrolysate + P_i ; and lane 5, whole cell hydrolysate.

Abbreviations: AEP, 2-aminoethylphosphonic acid (ciliatine); P_i , inorganic phosphate.

9) K. E. Kennedy and G. A. Thompson, *Science*, **168**, 989 (1970).

10) H. E. Carter and R. G. Gaver, *Biochem. Biophys. Res. Comm.*, **29**, 886 (1968).

11) J. R. Culbertson, *J. Protozoal.*, **13**, 397 (1966).

role of the phosphonates in these membranes is not yet clear, but their resistance to endogenous hydrolytic enzymes is highly suggestive of a protective function.

Nevertheless, we feel that the evidence now available supports the concept that phospho-

nates may have stabilizing roles in specialized cellular membranes.

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