E. Truehart, A. Hemmens, *Amer. J. Pathol.* **38**, 289 (1961); J. T. Prior and W. H. Hartman, *ibid.* **32**, 417 (1956).
3. J. E. Dunphy and K. N. Udupa, *Ann. Surg.* **144**, 304 (1956); S. M. Levenson, E. F. Geever, L. V. Crowley, J. F. Oates, C. W. Berard, H. Rosen, *ibid.* **161**, 293 (1965); E. Mussini, J. J. Hutton, S. Udenfriend, *Science* **157**, 927 (1967); see also W. Montagna and R. E. Billingham, Eds., *Advances in Biology of the Skin* (Pergamon, New York, 1964), vol. 5.
4. S. Udenfriend, *Science* **152**, 1335 (1966).
5. I. Lorenzen, *Acta Endocrinol.* **39**, 615 (1962).
6. N. Stone and A. Meister, *Nature* **194**, 555 (1962); J. J. Hutton, A. L. Tappel, S. Udenfriend, *Anal. Biochem.* **16**, 384 (1966).
7. R. O. Langner and G. C. Fuller, *Biochem. Biophys. Res. Commun.* **36**, 559 (1969).
8. T. Kobaysi, *Acta Pathol.* **76**, 193 (1969).
9. T. Takeuchi, K. I. Kivirikko, D. J. Prockop, *Biochem. Biophys. Res. Commun.* **28**, 940 (1967).
10. G. A. Gresham, A. N. Howard, A. J. King, *Brit. J. Exp. Pathol.* **43**, 21 (1962).
11. N. V. Perona, *Cor. Vasa* **8**, 148 (1966).
12. T. Hovig, L. Jorensen, M. A. Packham, J. F. Mustard, *J. Lab. Clin. Med.* **71**, 29 (1968).
13. A report of this data was presented at the Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, Pittsburgh, Pennsylvania, 1969 [R. O. Langner and G. C. Fuller, *Pharmacologist* **11**, 231 (1969)]. Supported by NIH grant HE 11578, the Rhode Island Heart Association, and an NDEA predoctoral fellowship awarded to R.O.L. We thank Mrs. N. Wood and Mrs. K. Mauger for technical assistance.
* Present address: Department of Pharmacology, School of Pharmacy and Pharmacy Research Institute, University of Connecticut, Storrs.
5 January 1970; revised 2 March 1970

Phosphonolipids: Localization in Surface Membranes of Tetrahymena

Abstract. *Approximately 60 percent of the phospholipids from the membrane sheath of* Tetrahymena pyriformis *cilia contain 2-aminoethylphosphonic acid. This is more than twice the concentration found in total cell lipids. The resistance of these lipids to hydrolytic enzymes suggests that they increase the stability of the surface membranes.*

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. The most widely distributed phosphonic acid is 2-aminoethylphosphonic acid (AEP), an analog of phosphorylethanolamine. 2-Aminoethylphosphonic acid occurs bound 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 enzymes (*2*) 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 Roberts (*1*) and by LaNauze (*4*). They postulated that the resistance of phosphonolipids to enzymatic breakdown may confer stability on the cellular membranes of which these molecules are a part. Such lipids might provide greater integrity to the outer membrane, especially where that membrane encounters hydrolytic enzymes, as in the case of rumen protozoa.

Our studies on membrane fractions from *Tetrahymena pyriformis*, a protozoan rich in AEP, have provided data which support suggestions of a protective role for the compound. We report here that surface membrane fractions of *Tetrahymena,* particularly membranes surrounding the cilia, contain higher percentages of phosphonolipids than do membranes within the cell.

Logarithmic phase cultures of *Tetrahymena pyriformis*, strain E, grown in 200-ml lots in enriched proteose-peptone medium (*5*) were deciliated according to the procedure of Rosenbaum and Carlson (*6*), modified so that suspensions of deciliated cells were diluted with only 2 volumes of growth medium instead of the 20 volumes used previously (*6*). Deciliated cell suspensions were centrifuged for 10 minutes at 650*g* and 2000*g* to remove whole cells and cell fragments, respectively. Cilia were then collected by centrifugation at 16,000*g* for 15 minutes. Electron micrographs showed that almost all detached cilia retained their membrane sheaths (Fig. 1) and that about 80 percent of the membranous material in the isolated fraction was associated with the cilia. Membranous contamination was due primarily to the presence of mitochondria arising from the few cells ruptured during the procedures. Ciliary phospholipids accounted for approximately 2.0 percent of the total cellular lipid phosphorus.

Table 1. Cellular distribution of lipid phosphonate in *Tetrahymena*. Figures in parentheses indicate number of experiments averaged.

Fraction	Phosphonate (% of total lipid phosphorus)	Average deviation
Whole cells	29.0 (4)	5
Crude mitochondria	32.2 (2)	0.5
Crude microsomal fraction	29.8 (3)	2
Cilia	63.0 (6)	7

Deciliated cells were disrupted in a Potter-Elvehjem homogenizer in 0.2*M* potassium phosphate buffer, *p*H 7.1, containing 0.1*M* NaCl and 3 m*M* ethylenediaminetetraacetate. Homogenates were centrifuged at 19,600*g* for 20 minutes to obtain crude mitochondria. The resulting supernatant was centrifuged at 100,000*g* for 60 minutes to obtain a microsomal fraction.

Lipids were extracted according to the method of Bligh and Dyer (*7*) and chromatographed on thin-layer silicagel G plates as described (*3, 5*). In labeling experiments, radioactivity in lipid fractions scraped from thin-layer chromatography (TLC) plates was assayed with a Packard Model 3310 scintillation spectrometer. Total phosphorus in lipid extracts was measured by the method of Bartlett (*8*) modified by digestion with perchloric acid according to Marinetti (*9*). Ester phosphorus was determined by the method of Aalbers and Bieber (*10*). Phosphonate phosphorus was calculated as the difference between total and ester phosphorus.

In repeated trials, 50 to 75 percent of the phosphorus in ciliary lipids was found as phosphonates (Table 1). The percentage of phosphonate phosphorus in whole cells and in isolated mitochondria and microsomes was 25 to 35 percent. Isolated pellicles seem to contain slightly higher amounts of AEP—up to 40 percent (*11*).

These findings were based on differential analyses of total lipid extracts, as described above. While such data do not imply which phosphonolipid is enriched, evidence on this point is provided by visual examination of TLC plates. In the case of cilia extracts, spots previously identified as 2-aminoethylphosphonolipid (*3*) were much darker, relative to other lipids, than in extracts of other membranes (Fig. 2) indicating a higher concentration of this compound.

The finding that phosphonolipids are resistant to degradation by lipolytic enzymes (*3*) suggested that the observed high percentage of phosphonate in ciliary lipids might be at least in part the consequence of selective breakdown

and loss of ester lipids during the isolation of cilia. To test this possibility, cells were incubated for 60 minutes with [^{14}C]palmitic acid, the incorporation of which is nearly complete after 5 minutes (*3*). At the end of 60 minutes, 85 to 90 percent of the radioactivity in lipid extracts of whole cells was contained in phospholipids. Assuming that all membranes attain a similar degree of incorporation, a drop in phospholipid radioactivity matched by a rise in [^{14}C]fatty acids can be considered a rough measure of how much enzymatic lipid breakdown is occurring.

Isolated mitochondria and microsomes showed a pattern of labeling resembling that of whole cells. The percentage of radioactivity in phospholipids of isolated cilia averaged 70 to 75 percent, an amount lower than that of whole cells. The percentage of label in free fatty acids, the usual degradation product, was not sufficient to account for this difference. At least 10 percent of the total radioactivity traveled on thin-layer plates to a position equal to or above triglycerides. Thus, hydrolysis by the usual pathway seems unlikely. Degradation by other enzymes may be occurring, or the cilia may contain a unique mixture of lipids.

To clarify this point, we conducted aging experiments in which fractions were incubated at 4°C for an additional 90 minutes after isolation. While microsomes sustained little lipid degradation, ciliary phospholipids showed as much as 20 percent breakdown over this period. The products of degradation included fatty acids as well as other more polar, uncharacterized compounds.

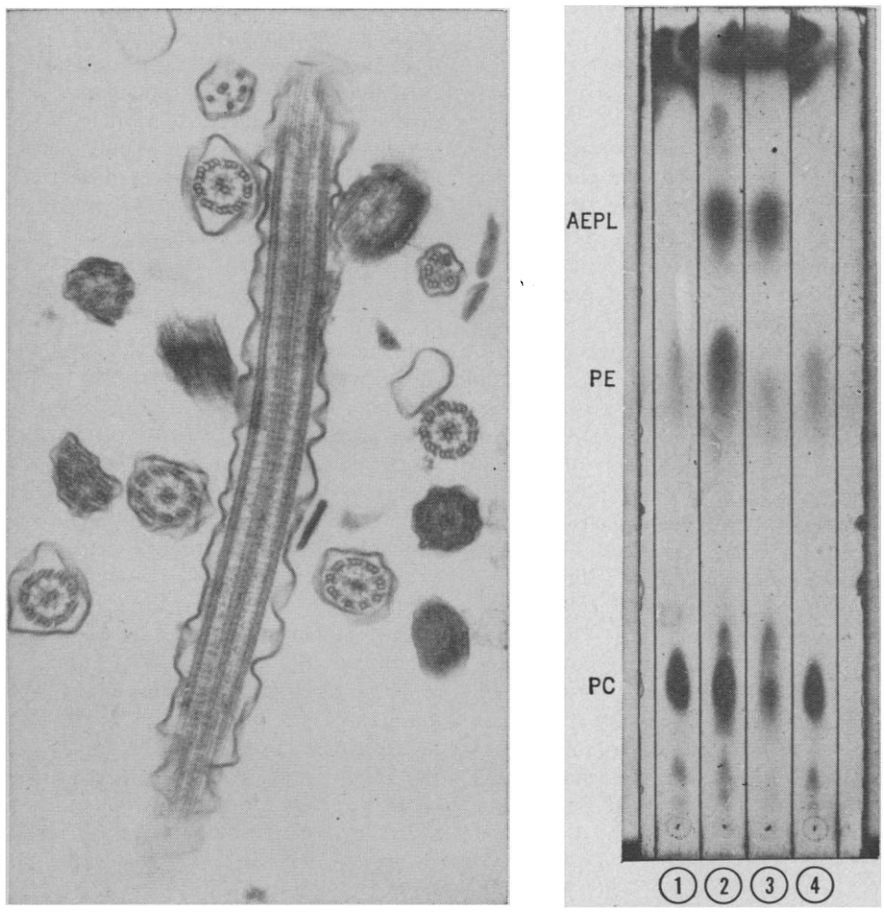

Fig. 1 (left). Electron micrograph of isolated cilia. The centrifuged pellet was resuspended with 2.5 percent glutaraldehyde in cold 25 m*M* sodium cacodylate buffer, *p*H 6.8. The isolated cilia were washed in buffer, embedded in 2 percent agar, and then fixed in cold 2 percent OsO_4 in cacodylate buffer. They were "block stained" in 0.5 percent uranyl acetate in 95 percent ethanol and embedded in Epon-Araldite (*14*). Sections were stained in uranyl acetate followed by lead citrate (× 20,350). Fig. 2 (right). Thin-layer chromatographic plate developed in a mixture of chloroform, acetic acid, methanol, and water (75 : 25 : 5 : 2.2 by volume) (*3*). Spots were made visible by spraying with H_2SO_4 and heating. Lane 1, egg lipids; lane 2, lipids from deciliated *Tetrahymena* cells; lane 3, lipids from *Tetrahymena* cilia; and lane 4, egg lipids. Abbreviations: *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; and *AEPL*, aminoethylphosphonate lipid.

Therefore efforts were made to minimize any degradation which may occur during isolation in order to measure more precisely phosphonate concentrations in vivo. Because Ca^{2+} activates some phospholipases (*12*), we conducted some experiments with the substitution of $MgCl_2$ for $CaCl_2$ in the deciliation procedure. Furthermore, the time required to isolate cilia was reduced to about 20 minutes instead of the usual 60 to 90 minutes. Centrifugation times were shortened and lipids were extracted from cilia while still in suspension. Phase contrast microscopy showed purity of cilia in these experiments to be comparable to that obtained by the standard procedure. With these modifications, the results obtained were not significantly different from those reported above.

Though our findings suggest that the phosphonate phosphorus content of cilia may indeed be somewhat elevated as a result of selective lipid degradation, analysis of the data shows unequivocally that degradation could account for only a small fraction of the observed phosphonolipid enrichment. In order for the percentage of phosphonate to increase from 30 percent (whole cells) to 60 percent (cilia) through loss by degradation of phosphate diester lipids, a destruction of about 70 percent of the diester lipids would be necessary. The observed difference in radioactivity in phospholipids of cilia and whole cells is only 10 to 20 percent (*13*).

Preliminary pulse-labeling experiments indicate that phosphonolipid metabolism proceeds at a slower rate than that of the other phospholipids. At intervals of 1, 5, or 60 minutes after a pulse of [^{14}C]palmitate, the total specific radioactivity of cell phosphonolipid is never more than 50 percent as high as that of ester phosphatides in membrane fractions. This is in agreement with earlier reports (*3*). In isolated cilia neither type of lipid ever approached the high specific radioactivities attained in other cell fractions in the time interval studied. The phosphonolipid specific radioactivity rose slowly and at approximately the same rate (relative to the rate for diester phospholipids) in all fractions.

Tetrahymena is the first organism reported to show a significant enrichment of phosphonolipids in its outer membranes. The role of the phosphonolipids in these membranes is not yet clear, but their resistance to endogenous hydrolytic enzymes is highly suggestive of a protective function. As has been re-

ported earlier (*5*), a large fraction of *Tetrahymena* phosphonolipids is further stabilized by the presence of an ether bond replacing the more easily hydrolyzed fatty ester linkage. The percentage of ciliary lipids so constructed has not yet been determined. Nevertheless, we feel that the evidence now available supports the concept that phosphonolipids may have stabilizing roles in specialized cellular membranes.

KATHLEEN E. KENNEDY
GUY A. THOMPSON, JR.
Department of Botany,
University of Texas, Austin 78712

References and Notes

1. J. S. Kittredge and E. Roberts, *Science* **164**, 37 (1969).
2. E. Roberts, D. S. Simonsen, M. Horiguchi, J. S. Kittredge, *ibid.* **159**, 886 (1968).
3. G. A. Thompson, Jr., *Biochim. Biophys. Acta* **176**, 330 (1969).
4. J. LaNauze, thesis, John Curtin School of Medical Research, Canberra, Australia (1969).
5. G. A. Thompson, Jr., *Biochemistry* **6**, 2015 (1967).
6. J. Rosenbaum and K. Carlson, *J. Cell Biol.* **40**, 415 (1969).
7. E. G. Bligh and E. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
8. G. R. Bartlett, *J. Biol. Chem.* **234**, 466 (1959).
9. G. V. Marinetti, *J. Lipid Res.* **3**, 1 (1962).
10. J. A. Aalbers and L. L. Bieber, *Anal. Biochem.* **24**, 443 (1968).
11. Details of pellicle isolation to be reported elsewhere.
12. G. B. Ansell and J. N. Hawthorne, *Phospholipids* (Elsevier, Amsterdam, 1964), chap. 6.
13. Data comparable to ours for phosphonolipid enrichment of cilia and pellicles have been obtained in this laboratory by Dr. Y. Nozawa using an entirely different deciliation procedure requiring only 15 minutes for isolation of fractions.
14. H. H. Mollenhauer, *Stain Technol.* **39**, 111 (1964).
15. Supported in part by NSF grant GB-6992, Robert A. Welch Foundation grant F-350, and the University of Texas Biomedical Sciences support grant. The technical assistance of Mrs. R. Derrenberger is gratefully acknowledged.

10 February 1970; revised 1 April 1970

Ribosomes from Spores of Bacillus cereus T

Abstract. *Data from analytical ultracentrifuge studies suggest that polymers of ribosomes exist in dormant spores of* Bacillus cereus *T.*

Kobayashi *et al.* (*1*) have suggested that spores of *Bacillus cereus* T do not contain a messenger ribonucleic acid (mRNA). Kornberg and co-workers have reported that patterns suggestive of polyribosomes are found in extracts from spores of *B. megaterium* (*2*). It is possible that the spores of these two species of *Bacillus* differ from one another in this significant respect, or that the results can be explained by the different methods used, or both. Here we present additional evidence (*3*) that some of the differences can be attributed to the methods used.

Lyophilized spores of *B. cereus* T were ground in a mortar with two times their mass of washed sand and then suspended in lysing buffer [20 ml per gram of spore; 10 m*M* tris(hydroxymethyl)aminomethane, *p*H 7.5; 100 m*M* NH_4Cl; 10m*M* magnesium acetate; 4 m*M* β-mercaptoethanol; and 1.0 m*M* ethylenediaminetetraacetic acid (EDTA)]. The suspensions were centrifuged twice at 40,000*g* at 0°C for 15 minutes to eliminate cell debris. Ribosomes were harvested from the supernatant by centrifugation in the 50 rotor of a Spinco model L ultracentrifuge at 42,000 rev/min for 30 minutes at −9°C. They were washed once with standard buffer (lysing buffer minus EDTA) by suspension and recentrifugation as before. The washed ribosomal pellet was suspended in standard buffer and analyzed in the Spinco model E analytical ultracentrifuge. All preparative steps were done between 0° and 4°C to minimize degradative enzymic reactions.

The peaks of ribosomes in Fig. 1A have sedimentation coefficients of 29.3, 47.6, 66.2, 94.2, 121.5, and 142.6*S*. The material in the centrifuge cell showed an absorbancy of 30 at 260 nm. An identical preparation was treated with pancreatic ribonuclease (1 μg/ml) for 5 minutes on ice just prior to loading the centrifuge cell. The sedimentation velocity pattern (Fig. 1B) illustrates the great sensitivity to ribonuclease of the bonds responsible for maintaining the multimer pattern.

Extraction with the nonionic detergent Brij 58 (1 percent weight to volume, final concentration) caused an increase in the yield of ribosomes from spores, but did not destroy the multimer profile, suggesting that membrane contamination did not contribute to the multimer patterns. These peaks were also sensitive to low concentrations of magnesium ion. No multimers were found when washed spore ribosomes were suspended in the absence of magnesium. As the concentration of magnesium increased from 2 to 5 m*M*, increasing amounts of the multimeric components were found. Above 5 m*M* magnesium ion, the pattern remained constant to at least 20 m*M* magnesium. Thus the probability that the patterns were artifactual aggregations due to divalent cation concentration is small.

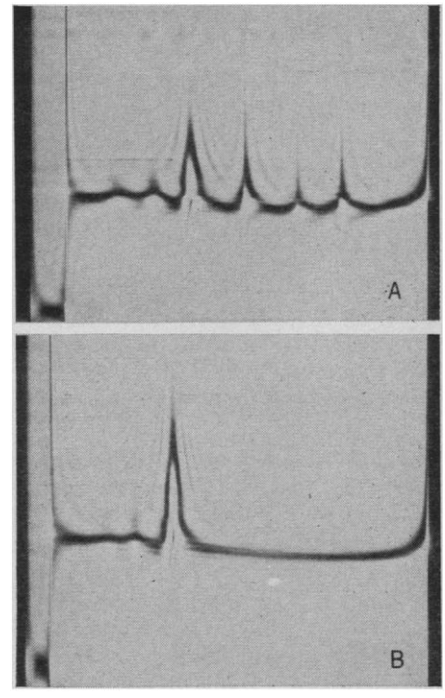

Fig. 1. Sedimentation velocity patterns obtained with ribosomes isolated from spores of *Bacillus cereus* T. Conditions of centrifugation were 40,000 rev/min; an D rotor; schlieren camera; analyzer angle, 35°; temperature of run, 20°C; and 4° sector standard kel-F centerpiece. (A) Ribosomes in standard buffer. (B) Ribosomes in standard buffer treated with pancreatic ribonuclease (1 μg/ml) for 5 minutes on ice. Sedimentation is from left to right. These pictures were taken 12 minutes after maximum speed was attained.

Ribosomal multimers are present in spores of *B. cereus* T. That they have not been seen previously is probably due to the difficulty in breaking dormant spores. The extent to which the presence of these multimers account for defects in protein synthetic characteristics of spores of this organism is still unknown.

FRED M. FEINSOD
H. A. DOUTHIT
Department of Botany, University of Michigan, Ann Arbor 48104

References

1. Y. Kobayashi, W. Steinberg, A. Higa, H. O. Halvorson, C. Levinthal, in *Spores III*, L. L. Campbell and H. O. Halvorson, Eds. (American Society for Microbiology, Ann Arbor, Mich., 1965), pp. 200–212.
2. P. Chambon, M. P. Deutscher, A. Kornberg, *J. Biol. Chem.* **243**, 5110 (1968); M. P. Deutscher, P. Chambon, A. Kornberg, *ibid.*, p. 5117.
3. J. M. Idriss and H. O. Halvorson, *Arch. Biochem. Biophys.* **133**, 442 (1969).
4. Supported in part by a grant from the University of Michigan Cancer Research Institute.

13 February 1970