

# STUDIES OF MEMBRANE FORMATION IN *TETRAHYMENA PYRIFORMIS*

## II. Isolation and Lipid Analysis of Cell Fractions

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### ABSTRACT

A method has been devised to fractionate cells of *Tetrahymena pyriformis*, yielding pure or highly enriched preparations of cilia, cilia-associated soluble material, pellicles, mitochondria, microsomes, and postmicrosomal supernatant. The method prevents the destructive action of lipolytic enzymes commonly associated with this organism. Analysis of the membrane lipids of these fractions reveals significant differences in lipid composition. Most noteworthy are the high concentrations of phosphonolipid and tetrahymanol in the surface membranes.

### INTRODUCTION

The ciliate *Tetrahymena pyriformis* is widely recognized as an organism well suited for the study of cell biology. Our laboratory has looked upon it as a potentially useful system for examining the mechanisms of biological membrane fabrication.

For this purpose it has certain important advantages. Unlike bacteria, *Tetrahymena* contains most of the subcellular organelles found in higher animal cells. The cells grow rapidly and incorporate precursors of membrane structural lipids with unusual speed (1). Thus it combines the experimental conveniences of a unicellular organism with the physiological makeup of a higher animal cell. The lipids are present in great variety, including considerable amounts of ether and phosphonate derivatives (1). Some of these are concentrated in certain organelles (2, 3), therefore qualifying as specific markers. One troublesome disadvantage is the presence of a system of hydrolytic enzymes which are subject to activation

upon disruption of the cells. After considerable difficulty we have discovered how to prevent these enzymes from degrading the subcellular organelles during the course of their isolation (4).

In this communication we describe procedures for separating a number of different *Tetrahymena* membrane fractions. The lipid composition of the fractions is reported. The use of these methods for the study of membrane biosynthesis is described in the accompanying paper (5).

### MATERIALS AND METHODS

*Tetrahymena pyriformis*, strain E, was cultured at 24°C in an enriched proteose peptone medium as previously described (1). 200-ml cultures were harvested after 39–42 hr, when the cells had reached middle logarithmic phase (approximately  $4-6 \times 10^6$  cells/ml). At this time, the cultures were quickly chilled to 4°C in an acetone:Dry Ice bath and centrifuged at 164 *g* for 5 min in the GSA rotor

of a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) maintained at 4°C. The cells were resuspended in 200 ml of 0.2 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl and 3 mM disodium ethylenediaminetetraacetate, and washed by another 5-min centrifugation at 100 *g*. Throughout the following procedure (Fig. 1), the cellular material was maintained in the above phosphate buffer, supplemented with sucrose as outlined.

The washed cells were resuspended in 12 ml of the cold buffer and gently homogenized by hand (4–6 strokes) in a loose-fitting glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) until most of the cilia had been removed from the cells as determined by phase microscopy. The completeness of deciliation depends upon the particular homogenizer used and the experience of the operator. The homogenate was then centrifuged at 1020 *g* for 5 min in a Sorvall HB-4 swinging bucket rotor to sediment deciliated cells. The supernatant was recentrifuged at 14,600 *g* for 5 min. The resulting white pellet of cilia was washed once with buffer.

The pellet of deciliated cells obtained by centrifugation at 1020 *g* was resuspended in 4.5 ml of buffer and homogenized vigorously by hand (80–100 twisting strokes) in a tight-fitting glass homogenizer (Arthur H. Thomas Co.) The homogenate was layered on a discontinuous buffered sucrose gradient (0.34 M, 10 ml; 1.0 M, 15 ml; 1.46 M, 15 ml) and centrifuged at 4080 *g* for 5 min. Three major zones were separated: Zone A, a top band down through the 0.34 M layer; Zone B, a discrete band of pellicles at the interface between the 1.0 M and the 1.46 M layers; Zone C, a very small pellet of unbroken cells at the bottom.

The pellicle fraction (Zone B) was removed carefully by syringe and centrifuged at 8000 *g* for 5 min to form a pellet. Lipid was extracted directly from the pellet.

Zone A, which was composed of mitochondria and less dense material, was diluted by one third of its volume of buffer. This suspension was spun at 19,600 *g* for 20 min to sediment mitochondria for extraction. The resulting supernatant was further subjected to centrifugation at 100,000 *g* for 60 min, in the SW-41 rotor of a Spinco L2-65B ultracentrifuge (Spinco Division of Beckman Instruments, Inc., Fullerton, Calif.), yielding a pellet comprised of microsomes. Both the microsomes and the post-microsomal supernatant were submitted to lipid extraction.

Lipids were extracted by the method of Bligh and Dyer (6). The final chloroform phase obtained by this method was washed two times by the method of Folch et al. (7) to remove any remaining traces of inorganic phosphate. Lipid extracts were stored in chloroform:methanol, 6:1 (v/v), at 4°C.

Silica Gel G thin-layer chromatography (TLC)

for neutral lipids utilized the solvent system petroleum ether:ethyl ether:acetic acid (70:30:1). Phospholipids were separated by using the solvents chloroform:acetic acid:methanol:water (75:25:5:2.2), a mixture modified from the work of Kapoulas (8). All solvents were mixed by volume.

Total phosphorus was determined by the method of Bartlett (9) modified by digestion with perchloric acid according to Marinetti (10). Ester phosphorus was measured by the procedure of Aalbers and Bieber (11), and phosphonate phosphorus was calculated as the difference between the total amount of phosphorus and that of ester phosphorus. The results were corrected for a 5% hydrolysis of phosphonate in the Aalbers and Bieber method. The estimation of phospholipid distribution on thin-layer plates of silica gel H was carried out by using a slight modification of the method of Rouser et al. (12).

Protein was measured by the method of Lowry et al. (13), using bovine serum albumin as a standard. Before analysis, samples were dialyzed for 12 hr at 4°C to remove any interfering material.

Samples were fixed for electron microscopy by resuspending the centrifuged pellet with 2.5% glutaraldehyde in cold 25 mM sodium cacodylate buffer, pH 6.8. The material was then washed in buffer, embedded in 2% agar, and fixed in cold 2% OsO<sub>4</sub> in cacodylate buffer. It was "block stained" in 0.5% uranyl acetate, in 95% ethanol, and embedded in Epon-Araldite (14). Sections were stained in uranyl acetate followed by lead citrate.

For the experiments involving radioisotopes, 1-<sup>14</sup>C-palmitic acid (54 mCi/mmole) obtained from New England Nuclear Corp. Boston, Mass., was introduced beneath the surface of the rapidly stirred culture in 2–4 drops of ethanol. Radioactive samples were counted in a Packard Model 3310 scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) The distribution of radioactivity among different lipids was determined after thin-layer chromatography by scraping areas of the plate into scintillation vials and assaying as described previously (1).

## RESULTS

### *Separation of Cell Organelles*

Many methods, both new and previously published (e.g. 15, 16, 17), were tested for the isolation of subcellular fractions from *Tetrahymena*. Some potentially useful procedures (18, 19) were not considered because they employ detergents or other surface active agents which, we feared, might result in exchange of lipid molecules from one membrane type to another during isolation. The technique finally adopted was chosen because

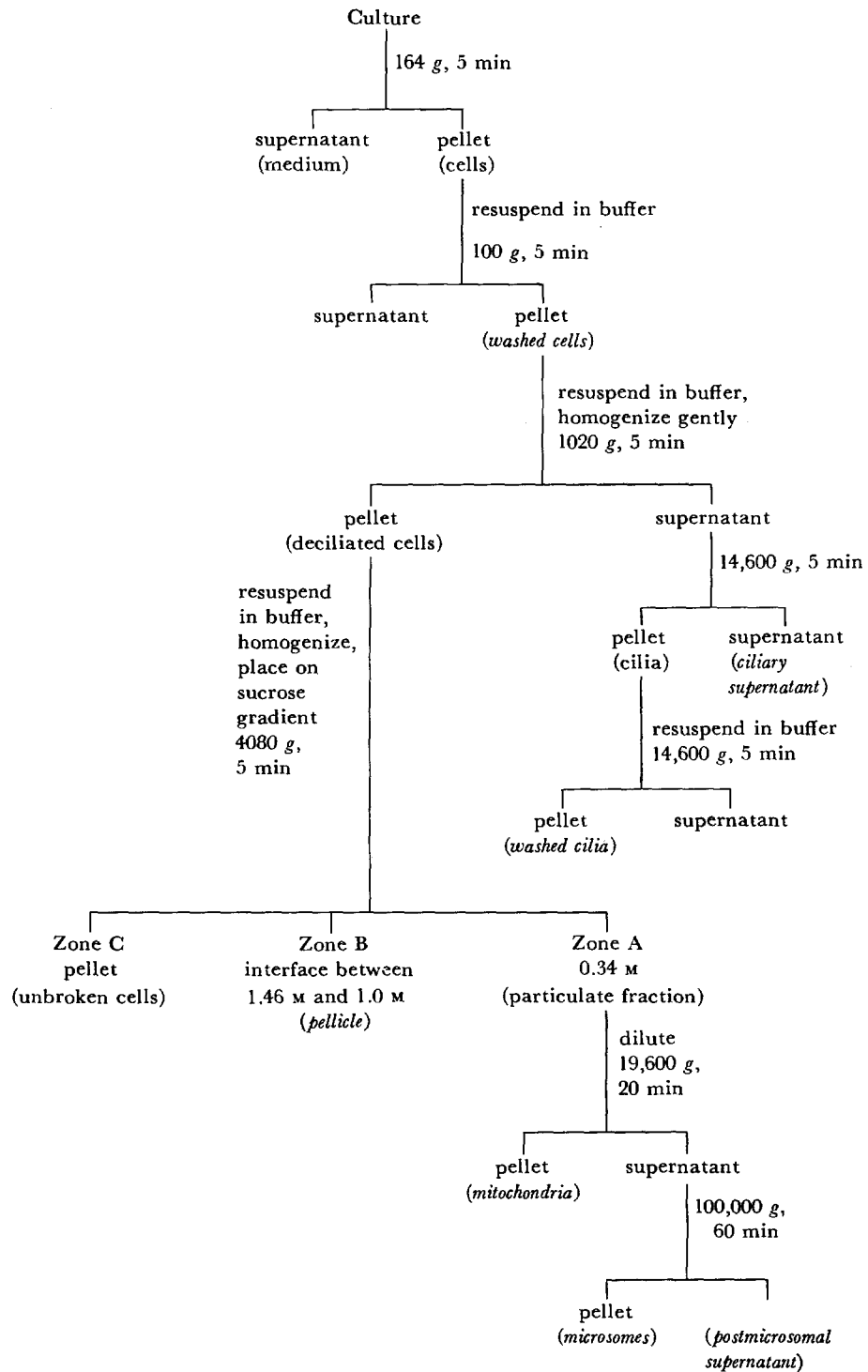


FIGURE 1 Scheme for the isolation of *Tetrahymena* subcellular fractions. For details, see the text.

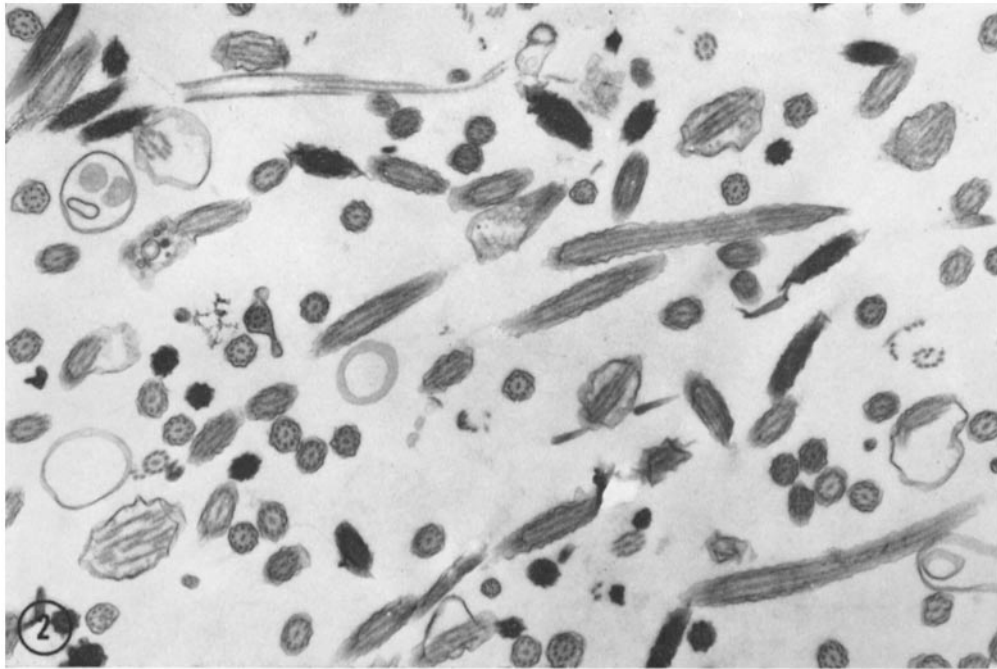


FIGURE 2 Typical field from pellet containing purified cilia.  $\times 13,200$ .

it was the only one found which preserved intact the complex of cell surface membranes known as the pellicle. The critical condition necessary for maintaining intact pellicles is the high ionic strength resulting from the use of 0.2 M phosphate buffer. The addition of 0.1 M NaCl confers further stability to the preparations, so that the pellicles will retain their normal appearance for many hours at 4°C.

In the homogenizing medium that we employ, deciliation is essentially complete (except for oral cilia) after 4–6 gentle strokes with the hand homogenizer, while many vigorous strokes (depending upon the individual homogenizer used) are necessary to produce a fissure in the pellicle large enough for the cytoplasm to be discharged. While most of the extruded cell particulate material remains intact, this process unfortunately leads to the destruction of the macronuclei, and the disposition of the nuclear fragments is not at present known.

Fig. 1 illustrates the steps involved in separating the various cell fractions. Because *Tetrahymena* has been shown to contain potent lipolytic enzymes (4), care must be taken to carry out the entire

procedure at 0°–4°C, and with no unnecessary delay. The time required to complete the procedures outlined in Fig. 1 is 120 min. Lipids are extracted from the first fractions, cilia and ciliary supernatant, within 15 min after deciliation. To test for possible lipid degradation during fraction isolation, a number of experiments were performed using cells prelabeled for 60 min with  $^{14}\text{C}$ -palmitate. Under the growth conditions employed, over 95% of the isotope is incorporated into lipids, mainly phospholipids (20). Extraction of the subcellular fractions after separation revealed little or no cleavage of  $^{14}\text{C}$ -fatty acids from the phospholipids. Radioactivity of free fatty acids in the isolated lipids generally ranged from 4 to 10% of the total.

The homogeneity of the subcellular fractions was measured by electron microscopy. The cilia were essentially devoid of identifiable contaminants (Fig. 2), indicating an important advance over the previously used method (2). The ciliary supernatant contains a surprisingly large amount of phospholipid. When this fraction is subjected to centrifugation at 100,000  $g$  for 60 min, approximately two thirds of the lipid is sedimented.

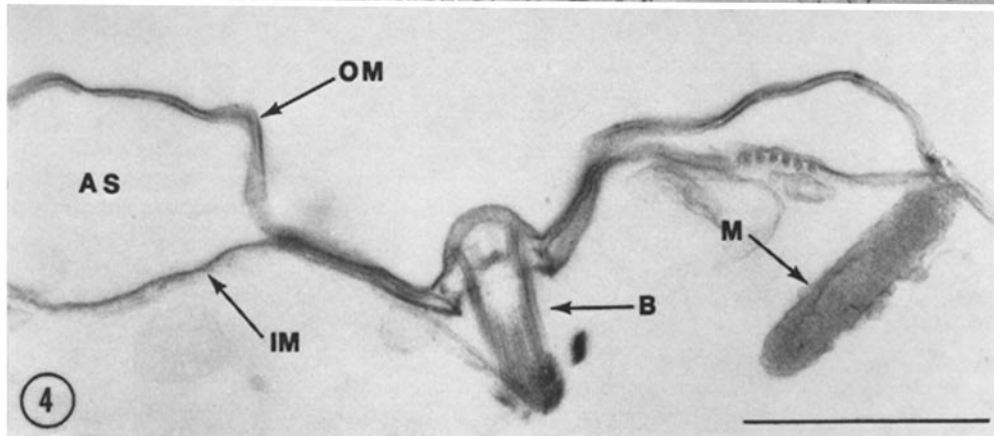
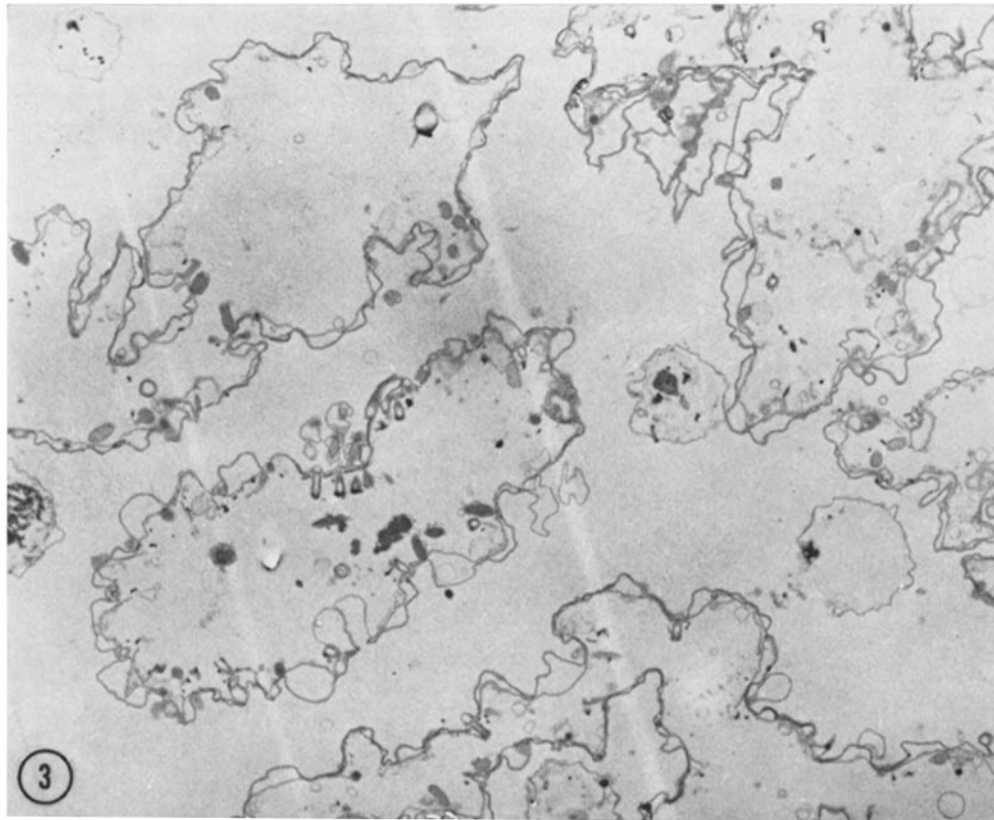


FIGURE 3 Low magnification field showing purified pellicle fraction.  $\times 5000$ .

FIGURE 4 Higher magnification view of pellicular fine structure. *OM*, outer membranes; *IM*, inner membrane; *B*, ciliary basal body; *M*, mucocyst; *AS*, alveolar space. The bar represents  $1 \mu$ .  $\times 33,800$ .

Electron microscopy of the pellet indicates a great enrichment in microtubules. The other major components are unidentified, membrane-enclosed, electron-opaque vesicles and fragments containing two unit membranes apposed as in the outer

pellicular membranes. Only a small amount of the remaining material sediments when this 100,000 *g* supernatant is recentrifuged for 60 min at 200,000 *g*.

The pellicles appeared to be almost free of

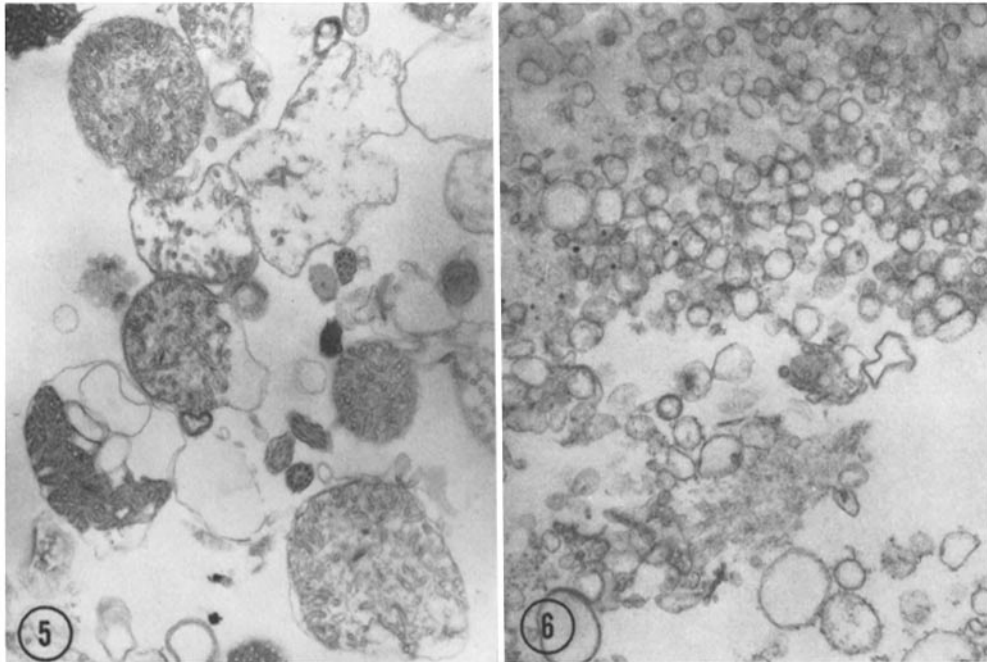


FIGURE 5 Mitochondrial fraction, showing contamination by cilia and other unidentified structures.  $\times 14,600$ .

FIGURE 6 Microsomal fraction, containing mainly smooth endoplasmic reticulum.  $\times 23,700$ .

contamination, as indicated by the typical field shown in Fig. 3. In all cases, the pellicles retained their normal organization of three adjoining unit membranes (Fig. 4). Kinetosome basal bodies and mucocysts (21) were also retained. Examination of unfixed pellicle preparations by phase microscopy showed that, except for a single large fissure per cell, the normal shape and appearance is preserved.

The mitochondrial fraction obtained by this procedure is grossly contaminated, principally by cilia (Fig. 5). The microsomal fraction (Fig. 6) is similar in appearance to microsomes from other cell types (22) and is almost free of identifiable contaminants.

#### *Analysis of Lipid Composition*

Analysis of the various fractions for lipid and protein are given in Table I. The protein content for whole cells is  $142 \mu\text{g}/10^6$  cells. The distribution of phospholipids shows considerable variation from fraction to fraction. Some of these differences are sufficiently large enough to be easily confirmed by

visual examination of thin-layer chromatograms (Fig. 7).

The data confirm the previously reported enrichment of ciliary membranes in phosphonolipid (2, 3). Two-dimensional TLC in the solvents chloroform:methanol:water (95:35:4) followed by chloroform:methanol: $\text{NH}_4\text{OH}$  (75:25:4) revealed the presence of considerable amounts of lysophospholipids in the cilia extracts. In contrast to the report of Smith et al. (3), phosphatidyl choline was always present in our cilia preparations, although it was sometimes partly masked by the lysophosphatides. When the phosphatidyl choline was freed from lysophosphatides by elution from an alumina column with chloroform:methanol (1:1), the purified lipid contained no more than 7% phosphonate phosphorus. The apparent discrepancy in phosphonate concentration suggested by data in the third and eighth columns of figures in Table I is therefore mainly accounted for by the presence of lyso-2-aminoethylphosphonolipid moving at and near the phosphatidyl choline zone.

TABLE I  
*Properties of the Phospholipids from Tetrahymena Cell Fractions*

| Cell fraction                 | Yield of phospholipid<br>(% of total recovered)* | Mg protein/mg<br>phospholipid | C-P bond (% of<br>lipid phosphorus)† | Mole % of total phospholipids (average of three determinations and range) |               |                          |               |               | Cardiolipin  |
|-------------------------------|--|-------------------------------|--------------------------------------|---|---------------|--------------------------|---------------|---------------|--------------|
|                               |  |                               |                                      | Lyso PC   | PC‡           | Lyso AEPL and<br>lyso PE | PE            | AEPL          |              |
| Whole cells                   | —  | 7.1                           | 29<br>(28-30)                        | 2<br>(1-3)  | 33<br>(28-42) | 0                        | 37<br>(33-39) | 23<br>(21-25) | 5<br>(3-8)   |
| Cilia                         | 4 ± 0.7  | 7.7                           | 67<br>(58-72)                        | 1<br>(0-3)  | 28<br>(27-36) | 9<br>(4-12)              | 11<br>(6-16)  | 47<br>(41-58) | 1<br>(0-2)   |
| Ciliary supernatant           | 3 ± 0.6  | 65.5                          | 44<br>(31-54)                        | 8<br>(3-12)   | 19<br>(16-20) | 13<br>(10-18)            | 16<br>(14-19) | 35<br>(34-38) | 1<br>(1-2)   |
| Pellicle                      | 22 ± 2.9   | 3.6                           | 42<br>(38-46)                        | 5<br>(3-6)  | 25<br>(21-31) | 3<br>(2-3)               | 34<br>(32-36) | 30<br>(26-32) | 2<br>(1-3)   |
| Mitochondria                  | 27 ± 3.5   | 7.5                           | 26<br>(25-30)                        | 2<br>(0-4)  | 35<br>(34-35) | 0                        | 35<br>(29-42) | 18<br>(17-18) | 10<br>(5-14) |
| Microsomes                    | 23 ± 3.0   | 2.2                           | 33<br>(29-39)                        | 1<br>(0-1)  | 35<br>(32-36) | 3<br>(1-6)               | 34<br>(30-41) | 23<br>(21-24) | 1<br>(0-2)   |
| Postmicrosomal<br>supernatant | 21 ± 3.6   | 13.5                          | 26<br>(21-34)                        | 5<br>(2-8)  | 34<br>(33-36) | 4<br>(3-8)               | 30<br>(26-33) | 22<br>(21-23) | 2<br>(1-4)   |

\* Mean of 10 experiments ± standard deviation.

† Determined by the method of Aalbers and Bieber (11); averages of at least three determinations and range.

‡ May contain lyso PE and lyso AEPL.

§ Data for the mole percentages of phospholipid components was obtained from thin-layer chromatography in which phosphorus recovery averaged 96%. The values are uncorrected for traces of minor constituents present. PC, choline phosphatides; PE, ethanolamine phosphatides; AEPL, 2-aminoethyl-phosphonolipid.

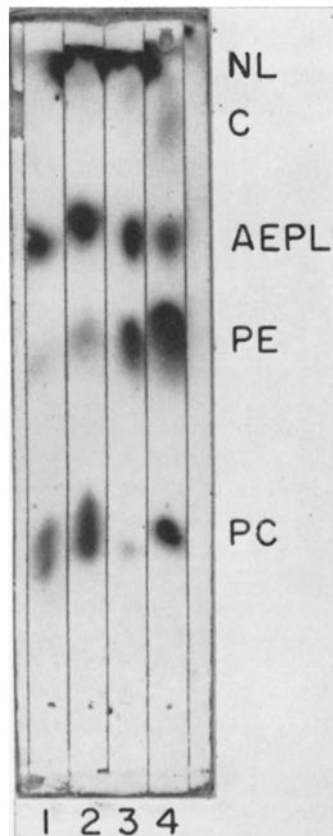


FIGURE 7 Thin-layer chromatogram developed in chloroform:acetic acid:methanol:water (75:25:5:2.2). 1, Cilia; 2, Ciliary supernatant; 3, Pellicle; 4, Mitochondria. *PC*, choline phosphatides (+ lyso PE); *PE*, ethanolamine phosphatides; *AEPL*, 2-aminoethylphosphonate lipids; *C*, cardiolipin; *NL*, neutral lipids.

Since the phosphonolipids are specifically associated with glyceryl ether derivatives in whole cell lipids (1), it was of interest to learn whether high concentrations of ether lipids are also characteristic of the ciliary membranes. The combined ciliary 2-aminoethylphosphonolipid and ethanolamine phospholipid purified on a silicic acid column was analyzed for its fatty acid ester content by infrared spectrophotometry (26). The calculated fatty acid ester/phosphorus ratio was 1.43, almost identical to the figure of 1.44 reported for the same lipid mixture from whole cells (1). However, these analyses must be repeated, and quantitative glyceryl ether assays must be carried out before the ciliary content of ether lipids can be known with certainty.

The "soluble" lipoprotein released during deciliation shows a phosphonolipid enrichment similar to that in cilia, and pellicles are intermediate in their phosphonolipid content. Because of its unexpectedly high lipid content, preliminary efforts have been made to characterize further the postmicrosomal supernatant fraction. When this fraction is submitted to centrifugation at 200,000 *g* for 60 min, equal amounts of phospholipid are recovered in the pellet and the 200,000 *g* supernatant. No marked differences can be detected in the lipid composition of the two samples. The pellet has not been examined by electron microscopy.

The triterpenoid tetrahymanol, which in this organism replaces sterols as the principal membrane neutral lipid, also varies in amount from one cell fraction to another. While a reliable

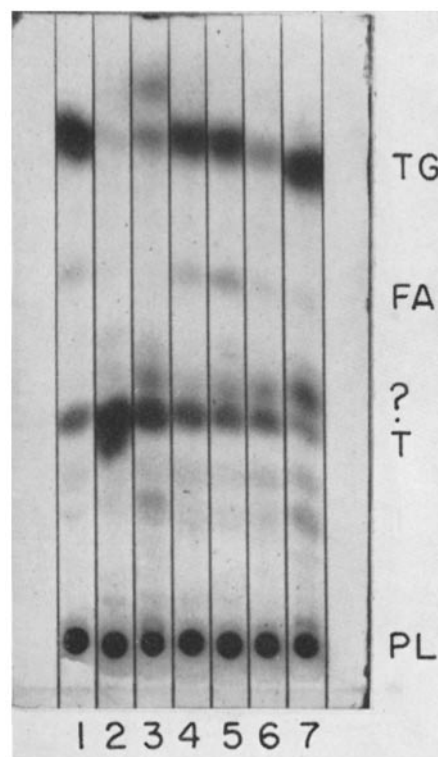


FIGURE 8 Thin layer chromatogram developed in petroleum ether:ethyl ether:acetic acid (70:30:1). Total lipid containing 10  $\mu$ g lipid phosphorus was applied to each lane. 1, whole cells; 2, cilia; 3, ciliary supernatant; 4, pellicles; 5, mitochondria; 6, microsomes; 7, postmicrosomal supernatant. *PL*, phospholipid; *T*, tetrahymanol; *FA*, fatty acids; *TG*, triglycerides.



method for quantifying this component has not yet been developed, visual comparison of the TLC patterns of neutral lipids isolated from the cell fractions (Fig. 8) reveals certain obvious differences. The tetrahymanol content is clearly much higher in relation to the phospholipid level in ciliary membranes than in other organelles. The ciliary supernatant also appears to be somewhat enriched in tetrahymanol. Labeling data discussed in the accompanying paper corroborate the visual evidence for tetrahymanol localization.

#### DISCUSSION

It has been recognized for some years that within a given cell the membranes of subcellular organelles may differ in their lipid composition. Mitochondria are generally enriched in cardiolipin (23), and the level of sterols is usually higher in plasma membranes than in cytoplasmic membranes (24). More recently, less striking but still significant differences have been reported in the intracellular distribution of other membrane lipids (25). It is widely believed that such dissimilarities may provide a useful clue as to how membranes are fabricated and what interrelationships exist between functionally different membrane systems.

The various membranes of *Tetrahymena* exhibit what is perhaps the most diversified phospholipid distribution yet described. Some of the differences, such as the cardiolipin enrichment in mitochondria, follow the pattern previously observed in other cell types. The most astonishing patterns are those of the ciliary membranes and their associated fraction. The high phospholipid level reported earlier (2) has been confirmed by the use of a new and more efficient isolation procedure. Although speculations have been made (2) regarding the physiological significance of this phospholipid enrichment, the mechanism by which it is achieved remains obscure. The biogenesis of the ciliary membranes is of particular interest because of their relative isolation from the metabolic mainstream of the cell. The structure of the *Tetrahymean* pellicle is such that access to the cilia by large cytoplasmic molecules such as proteins and complex lipids may be gained only by passage through the narrow openings through which the kinetosome basal bodies are jointed to the ciliary shaft (21). Over the greatest part of the cell surface of *Tetrahymena* and other ciliate protozoa, the plasma membrane, of which the ciliary membrane is an extension, is

separated from the cytoplasm by two intervening unit membranes which enclose the so-called alveolar space (21) (see also Fig. 4). We feel that this barrier to communication with the cell interior may be in some way responsible for the striking differences in lipid composition.

If the barrier does function in this manner, one would expect that the outermost of the three pellicular membranes would also be rich in phospholipid. This cannot be determined at the present time since it is not possible to subfractionate the pellicle into its component parts. However, calculations can be made based on the assumption that the outermost membrane is similar in phospholipid composition to the ciliary membrane and that the inner two are similar to the microsomal membranes. The calculated per cent phospholipid is 42, identical to the observed figure for the isolated pellicle. A similar agreement is reached in radioactivity distributions from isotope experiments described in the accompanying paper (5).

Tetrahymanol, the sterol-like lipid of this organism, also exists at an elevated level in the surface membranes. In this case, however, an explanation based upon the relative isolation of these membranes is less tenable, since numerous instances are known in other cell types of sterol enrichment in plasma membranes having direct contact with the cytoplasm (24).

We feel that *Tetrahymena* provides a unique system for the study of membrane assembly. Much additional work is called for to effect the separation of organelles not now recovered, such as nuclei, lysosomes, and vacuoles. The homogeneity of the isolated cell fractions must be measured by the use of marker enzymes and other criteria in addition to electron microscopy. Nevertheless, the separation scheme in its present stage of development offers a rapid means of obtaining several highly enriched fractions without appreciable lipid degradation. The accompanying communication describes our first efforts to exploit the potential of this procedure.

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