Further Studies of the Lipid Composition and Biochemical Properties of *Tetrahymena pyriformis* Membrane Systems*

Guy A. Thompson, Jr., Raymond J. Bambery, and Yoshinori Nozawa

ABSTRACT: Each of several membrane systems of *Tetrahymena* has been found to have a characteristic lipid distribution. The triterpene alcohol tetrahymanol is present in surface membranes at a concentration more than seven times that found in the cell's endoplasmic reticulum. The surface membranes also contain a threefold enrichment in alkyl glyceryl ether phospholipids. However, it appears that the lipid mixture arriving at these surface locations from the sites of their synthesis has not yet become enriched in these species. Possible mechanisms for achieving the selective accumulations are discussed.

Functionally distinct membranes within a particular cell often have markedly different lipid compositions. Early indications to this effect have recently been confirmed with the aid of improved procedures for cell fractionation and lipid analysis (Fleischer and Rouser, 1965; Coleman and Finean, 1966; Weinstein *et al.*, 1969). Among the more frequently reported differences in animal cells are the localization of cardiolipin in mitochondrial membranes and the enrichment of cholesterol in plasma membranes.

The localization of certain lipids in particular membranes is especially striking in the protozoan *Tetrahymena pyriformis*. Phosphonolipids, which have in addition to their characteristic phosphorus–carbon bond a high incidence of 1-*O*-alkyl ether linkages, are twice as prevalent in the surface membranes as in internal membranes (Kennedy and Thompson, 1970; Smith *et al.*, 1970; Jonah and Erwin, 1971; Nozawa and Thompson, 1971a). Another structural lipid, the triterpenoid tetrahymanol, is also present in a very high concentration in the outer membranes (Nozawa and Thompson 1971a).

In this report, we present more detailed information concerning the localization of various structural lipids in surface membranes. A new, highly sensitive gas chromatographic procedure for quantitative tetrahymanol analysis reveals marked differences in the level of this lipid. Alkyl glyceryl ether derivatives are also shown to differ from one cell fraction to another. In addition, we discuss a number of possible mechanisms whereby *Tetrahymena* may achieve this specific enrichment.

Materials and Methods

Cultures and Isolation of Lipids. Tetrahymena pyriformis, strain E, is cultured at 24° in an enriched proteose peptone medium with stirring as previously described (Thompson, 1967). Cultures (200 ml) are harvested 34–36 hr after innoculation, when cells have reached middle logarithmic phase (approximately 5×10^5 cells/ml). The cultures are quickly chilled to 4° in an acetone–Dry Ice bath, and the chilled cells are washed, homogenized, and separated into the six subcellular fractions as described previously (Nozawa and Thompson, 1971a). Lipids are extracted by the method of Bligh and Dyer (1959), and the resultant lipid solutions are stored in chloroform–methanol (6:1, v/v) at 4°. Lipid phosphorus is determined by the method of Bartlett (1959), modified by digestion with 70% perchloric acid according to Marinetti (1962). The lipid phosphorus content provides a basis for comparison of the tetrahymanol and glyceryl alkyl ether content of each subcellular fraction. Glyceryl alkyl ethers were isolated by submitting lipid (0.1–2.0 μmoles of lipid phosphorus) to acetolysis and saponification (Hanahan and Watts, 1961).[1] Glyceryl ethers freed in this manner were purified by column or preparative thin-layer chromatography and quantified by use of the periodate oxidation method (Thompson and Kapoulas, 1969).

Analysis for Tetrahymanol. An aliquot of total lipids equivalent to 5–50 μg of tetrahymanol is pipetted into a 13-ml centrifuge tube with Teflon-lined cap along with a convenient aliquot of β-amyrin standard (Koch-Light Laboratories) of from one-half to two times the estimated amount of tetrahymanol. The solvents are removed under a stream of nitrogen gas and then 1 ml of 2 N potassium hydroxide in 50% ethanol–water is added. The tube is capped tightly and the lipids are hydrolyzed on a boiling water bath for 2 hr.

After cooling, 3 ml of benzene is added to the tube. Following a vigorous mixing of the layers with a Pasteur pipet, the tube is centrifuged at 1500*g* in a clinical centrifuge for 5 min or until both layers are clear. The upper layer is then transferred by the Pasteur pipet into a small separatory funnel containing 15 ml of water. This extraction is repeated on the lower layer one more time, and finally a third extraction is made by adding first 3 ml of benzene and then 1 ml of water.

The water layer is then drained from the separatory funnel, and the benzene layer is washed one time with 15 ml of water. In this separation, it is not necessary for the benzene layer to

* From the Department of Botany, University of Texas, Austin, Texas 78712 (G. A. T. and R. J. B.), and from the Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu, Japan (Y. N.). *Received June 14, 1971.* Presented in part before the 62nd Annual Meeting of the American Oil Chemists Society, May 2–6, 1971, Houston, Texas, Abstract 179, and 62nd Annual Meeting, American Society of Biological Chemists, June 13–18, 1971, San Francisco, Calif., Abstract 370. Paper 4 of the series Studies of Membrane Formation in *Tetrahymena pyriformis*. Paper 3 is Nozawa and Thompson (1971b).

[1] It was found advisable to extend the refluxing time for the acetolysis reaction to 12 hr and the time for the saponification reaction to 3 hr.

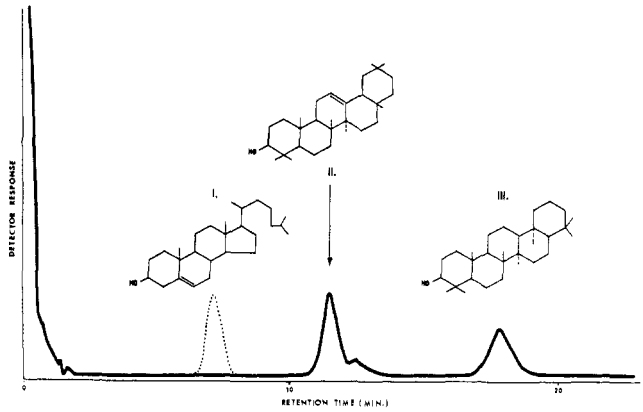

FIGURE 1: Gas–liquid chromatogram showing retention times for cholesterol (I), β-amyrin (II), and tetrahymanol (III). Operating conditions are given in Materials and Methods section.

be clear, but the water layer should be. The benzene layer is quantitatively transferred to a round-bottom flask, and absolute ethanol is added dropwise to the flask until any water droplets are solubilized. The solvents are removed on a rotary evaporator, and the hydrolysis products are transferred to a 15-ml centrifuge tube with four 2-ml portions of benzene. The benzene is then evaporated under a stream of nitrogen gas, and the sides of the tube are washed down one final time with 0.5 ml of benzene, which is likewise evaporated.

To this dried mixture is added 50 μl of benzene. The sample (10 μl) is injected onto a 6-ft glass column packed with 3% SE-30 on Gas Chrom Q, 80–100 mesh (Applied Science Laboratories), in a Barber-Colman Series 5000 gas chromatograph equipped with a flame ionization detector. The operating parameters are: column temperature, 268°; injector temperature, 280°; detector temperature, 284°; nitrogen carrier gas pressure, 18 psi; hydrogen pressure, 20 psi; and air pressure, 19 psi. Under these conditions, the retention time for β-amyrin is about 16 min and for tetrahymanol about 25 min.

Areas are calculated by means of the peak height times the width at half-height method according to Ball *et al.*, (1968). To ensure reliability, a second injection was made using proportionately less solvent (four-fifths) and the results of the two ratios of areas are averaged together.

Radioactive Isotope Incorporation. Sodium acetate-*1*-^{14}C (56.0 mCi/mmole) and palmitic acid-*1*-^{14}C (54 mCi/mmole) were purchased from New England Nuclear Corp. Tritium labeled chimyl alcohol was prepared as previously described (Thompson, 1965). Tetrahymanol-*1*-^{14}C was obtained by preparative thin-layer chromatography of lipids from *Tetrahymena* grown in the presence of sodium acetate-*1*-^{14}C.

Incubations with the radioactive tracers were carried out as described earlier (Nozawa and Thompson, 1971b), and the general labeling patterns were as reported in that publication.

Results

Quantitative Analysis of Tetrahymanol. Marked differences in tetrahymanol content in logarithmic phase *Tetrahymena* subcellular fractions have previously been shown by comparison of thin-layer chromatographic patterns (Nozawa and Thompson, 1971a). It seemed desirable to estimate the tetrahymanol levels in a more quantitative fashion. Because tetrahymanol does not give a Liebermann–Burchard color reaction, we have developed a method for its determination by gas–liquid chromatography.

The basic approach involves the use of an internal standard, which is added in a known amount to the lipid sample to be analyzed. The sample is then saponified, and the extracted saponification products are examined by gas–liquid chromatography. Because the unusual solubility properties of tetrahymanol gave rise to unexpected problems in achieving quantitative results, the procedures will be described in some detail.

Cholesterol initially seemed the obvious choice as an internal standard, since it is related structurally as well as functionally to tetrahymanol. However, cholesterol was found to have a much shorter retention time (Figure 1), and its solubility properties in the extracting solvents differ significantly from those of tetrahymanol. The more structurally similar triterpenoid, β-amyrin, does resemble tetrahymanol in its extraction properties, yet it is completely separable from tetrahymanol under the chromatographic conditions employed (Figure 1). Although the commercial preparation of β-amyrin contained a contaminant (presumably α-amyrin (Ikekawa, 1969)), producing a shoulder peak with a slightly longer retention time, it was a constant value which could be corrected for in the computation of peak areas.

The initial problem in quantification was to compare the detector response to the two compounds injected in different ratios. This was tested by chromatographing known mixtures in various weight ratios (tetrahymanol:β-amyrin) ranging from 0.376 to 2.257. In all cases the detector response mirrored the weight ratio, with an average agreement of 99.3%.

Problems of incomplete tetrahymanol recovery were encountered when known ratios of tetrahymanol and β-amyrin were subjected to saponification and solvent extraction by standard procedures. Exhaustive extraction with petroleum ether removed β-amyrin easily, but was unsatisfactory for the removal of tetrahymanol. (The possibility that tetrahymanol is incompletely extracted by hexane has been previously noted (Shorb *et al.* 1965).) The use of ethyl ether resulted in a higher tetrahymanol recovery, but the yields were still erratic (101 ± 27%), in our hands. Benzene proved to be the most desirable extracting solvent. Initial trials resulted in a 109 ± 12% recovery of tetrahymanol. Further modification of the procedure led to a greater precision. For instance, we discovered that if water is added to the hydrolysis mixture before the addition of benzene, a poorly extractable tetrahymanol precipitate forms on the walls of the tube. Conversely, if no water at all is added β-amyrin seems to be incompletely extracted. The best results are obtained when water is added after the first benzene extraction.

In a final check of the procedure, tetrahymanol and β-amyrin in known ratios were subjected to the entire isolation procedure in the presence of appropriate quantities of egg phospholipids in order to simulate as nearly as possible the conditions of the actual analyses to be performed. The results presented in Table I show that the procedure yields an average of 106.0 ± 3.0% of the tetrahymanol initially present before hydrolysis and extraction.

When lipids from the individual cell fractions were subjected to analysis by this procedure, the marked differences noted earlier by visual examination of thin-layer chromatograms were confirmed (Table II). The lipids extracted from cilia, ciliary supernatant, and pellicles, all of which are associated with the surface structures of the cell, contain tetrahy-

TABLE I: Gas Chromatographic Recovery of Tetrahymanol.[a]

Tetrahymanol: β-Amyrin Ratio Employed	Total Tetrahymanol before Anal. (μg)	Total Tetrahymanol Recovd (μg)
0.338	6.3	6.9
0.513	9.6	10.2
0.683	12.7	13.8
1.064	19.8	20.4
1.431	26.6	27.4

[a] As determined by comparison with a β-amyrin internal standard. See text for details.

manol in amounts exceeding those measured for the internal membranes.

Thin-layer chromatographic analysis, using benzene as the developing solvent and authentic tetrahymanol palmitate as a standard marker, indicates that in the logarithmic-phase cells used for these studies, essentially all the tetrahymanol occurs free. Significant amounts of fatty acyl esters of tetrahymanol have been reported in stationary-phase cells by Jonah and Erwin (1971).

Quantitative Analysis of Glyceryl Alkyl Ether Lipids. We have reported earlier (Nozawa and Thompson, 1971a) that in ciliary membranes phosphonolipids account for 67% of the total phospholipids—over twice the concentration found in microsomes. Because alkyl ether groups of whole cell lipids have been shown to exist rather specifically in those molecules which also contain a phosphonate base (Thompson, 1967), we proceeded to analyze all fractions for ether lipids. An analysis of the various cell fractions (Table III) revealed that alkyl ethers are indeed localized in the surface membranes. Other fractions of the cell contain a much lower concentration of ether lipids, with the lowest level being found in microsomes.

A systematic analysis of alkyl ether distribution among the various lipids of the membrane fractions has not been made. However, in the case of cilia we have analyzed some properties of the silicic acid column eluate which contains phosphonolipid plus phosphatidylethanolamine. Infrared spectrophotometry (Thompson and Kapoulas, 1969) of this fraction indicates that 60% of the molecules contain ether-linked side chains.

Further evidence for the specific association of alkyl ethers and 2-aminoethylphosphonate in the same molecule comes from experiments involving cells grown in the presence of chimyl-*t* alcohol. This alkyl ether is incorporated intact into phospholipids (Thompson, 1967). In all cell fractions radioactivity was recovered primarily in the phosphonolipid.

Intracellular Differences in Lipid Metabolism. The discovery of phosphonolipid, tetrahymanol, and alkyl glyceryl ether localization in the surface membranes of *Tetrahymena* raises the question of how such accumulations are brought about. Experience with a variety of cell types which, like *Tetrahymena*, have clearly defined endoplasmic reticulum indicates that most lipid biosynthetic enzymes occur in the endoplasmic reticulum fraction (Jungalwala and Dawson, 1970). The differences in lipid composition observed in the various cell organelles of *Tetrahymena* presumably arise either through a specificity in the system for transporting newly made lipids

TABLE II: Tetrahymanol Content of Whole Cells and Subcellular Fractions.

Cell Fraction	Tetrahymanol (μg/μg of Phosphorus) Expt 1[a]	Tetrahymanol (μg/μg of Phosphorus) Expt 2[a]	Mole Ratio Tetrahymanol: Phospholipids (Av)
Whole cells	0.853	0.727	0.057
Cilia	4.164	4.237	0.30
Ciliary supernatant	2.665	1.632	0.16
Pellicles	1.175	1.165	0.084
Mitochondria	0.625	0.717	0.048
Microsomes	0.481	0.646	0.041
Postmicrosomal supernatant	0.210	0.248	0.016

[a] Each figure is the average of duplicate chromatographic determinations. All results are divided by 1.06 to correct for spuriously high tetrahymanol recovery (see text).

TABLE III: Glyceryl Ether Content of Lipids from Isolated Cell Fractions.

Fraction	Glyceryl Ethers (Moles/100 Moles of Lipid Phosphorus) ± Std Dev
Whole cells	29.7 ± 5.4 (4)[a]
Cilia	52.6 ± 8.4 (5)
Ciliary supernatant	23.1 ± 4.4 (4)
Pellicles	32.8 ± 4.8 (3)
Mitochondria	24.7 ± 6.6 (3)
Microsomes	18.3 ± 8.7 (4)
Postmicrosomal supernatant	27.4 ± 2.0 (4)

[a] Figures in parentheses denote the number of analyses.

from the endoplasmic reticulum (our microsome fraction) to their particular membrane destinations or through a specificity at each membrane site allowing the retention of certain incoming lipid species and the rejection of others.

Evidence pertinent to this question has been obtained by extending previous observations on the intracellular redistribution of radioactive lipids (Nozawa and Thompson, 1971b). That study revealed that newly formed lipids rapidly enter cytoplasmic membrane fractions while cell surface membranes require several hours to acquire an equivalent specific radioactivity. Subsequent work (Nozawa and Thompson)[2] has shown that the distribution of radioactive lipids to the comparable membrane fractions of nongrowing cells[3] is only slightly less rapid than it is in logarithmically growing cells. Thus the movement of new lipids into the membranes of growing cells may actually be considered as consisting of

[2] Unpublished observations, 1971.

[3] Cells in the stationary growth phase or in minimal medium (Hamburger and Zeuthen, 1957).

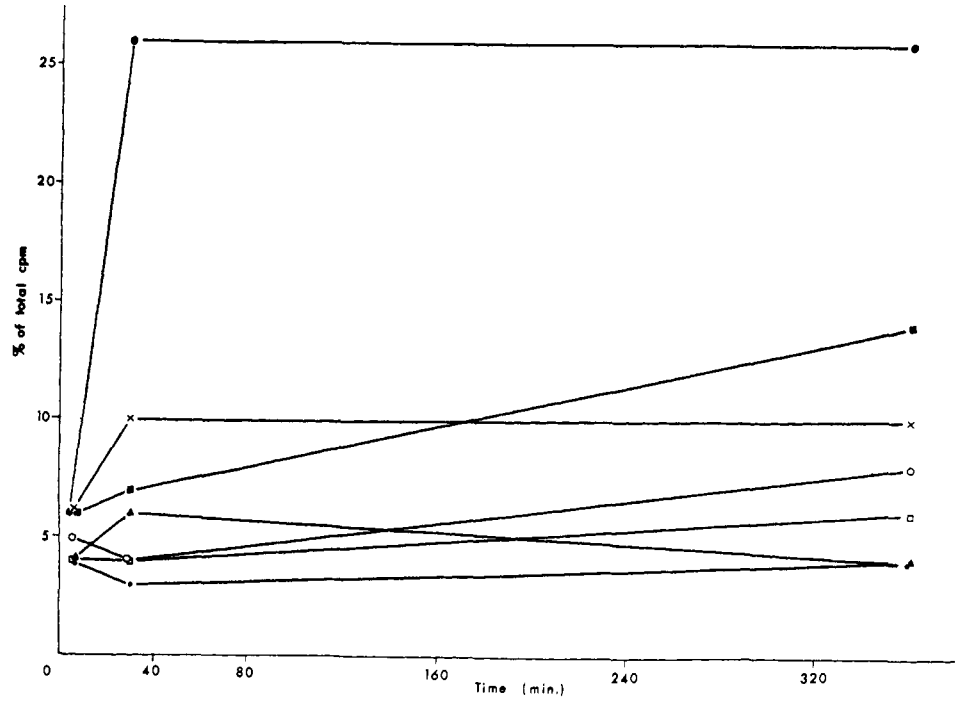

FIGURE 2: Percentage of total lipid radioactivity of isolated cell fractions that is found in tetrahymanol at various times after pulse-labeling cells with acetate-*1*-^{14}C. The different curves represent lipids of the following cell fractions: (·) post-microsomal supernatant, (□) microsomes, (○) mitochondria, (▲) whole cells, (■) ciliary supernatant, (×) pellicles, and (●) cilia.

two processes: first, the net addition of lipid necessary for membrane expansion, and second, an exchange of new molecules for old. Similarities in the labeling of growing and nongrowing cells suggest that the latter exchange process contributes greatly to the observed intracellular lipid mobility.

In effect, the surface membrane lipids are in a virtual steady state equilibrium with those of internal membranes, with the net lipid movement being zero in nongrowing cells and unbalanced toward the direction of surface membranes in growing cells. The novel feature of *Tetrahymena* surface membranes is that their physical isolation markedly increases the time of travel from site of lipid synthesis to site of deposition.

By analyzing the distribution of lipid radioactivity in surface membranes at various times, beginning immediately after administration of the isotope, one measures initially the assortment of lipids that first reaches these membranes and, later, the pattern of radioactivity resulting from the continuing accumulation and exchange of labeled lipids. We have made such analyses of ciliary membranes using several radioactive precursors, including palmitic-^{14}C acid, sodium acetate-^{14}C, and chimyl-*t* alcohol. In all cases the labeling patterns after 5 min resembled not the quantitative distribution of lipids in cilia but rather the quantitative lipid distribution in the microsomes and other internal fractions. However, as additional time elapsed following administration of the isotope, its relative distribution in cilia changed, so that the radioactivity was eventually apportioned in just the same pattern as is the mass of the lipids.

One example of this changing lipid distribution pattern in cilia and other membrane fractions was presented earlier (Nozawa and Thompson, 1971b). Graphing the radioactivity in the three major phospholipids of palmitate-^{14}C-labeled cells revealed that in cilia (and, to a much lesser degree, in all other fractions) there was a gradual rise in the relative labeling of the phosphonolipid and a concomitant drop in the labeling of phosphatidylcholine and phosphatidylethanolamine. The same general trends have been noted in experiments using acetate-^{14}C and chimyl-*t* alcohol. The data from the acetate-^{14}C pulse-labeling experiments have been plotted in a fashion illustrating the time-dependent ^{14}C accumulation in tetrahymanol of the surface membrane fractions. Although tetrahymanol is nearly 20 times more concentrated in cilia than in postmicrosomal supernatant, after a 5-min incorporation this lipid contains essentially the same percentage of the total radioactivity in both fractions (Figure 2). However, with the passage of time, the labeling patterns of ciliary membranes and other outer cell fractions change dramatically and soon become a reflection of the tetrahymanol concentrations (Table II). Similar data describing the appearance of administered chimyl-*t* alcohol in phosphonolipid are given in Figure 3. Here too, the labeling pattern does not reflect the widely divergent levels of phosphonolipid found in the different fractions until considerable time has elapsed. In both Figures 2 and 3, the continued slow rise of labeling in the internal membranes proves that accumulation in the surface membranes is not due to a specific transfer of that lipid from other fractions.

These findings indicate that all cellular membranes receive a similar assortment of lipids from the sites of synthesis, and that the pattern may subsequently be altered at the membrane itself. The means by which this is accomplished has not yet been deduced. However, a possible mechanism was suggested by *in vitro* studies we have made using the various purified membrane fractions.

When the fractions isolated from palmitate-^{14}C-labeled cells were incubated at room temperature for 3 hr, there was considerable phospholipid degradation by the phospholipase enzymes described previously (Thompson, 1969). The degradation was most pronounced in the mitochondrial

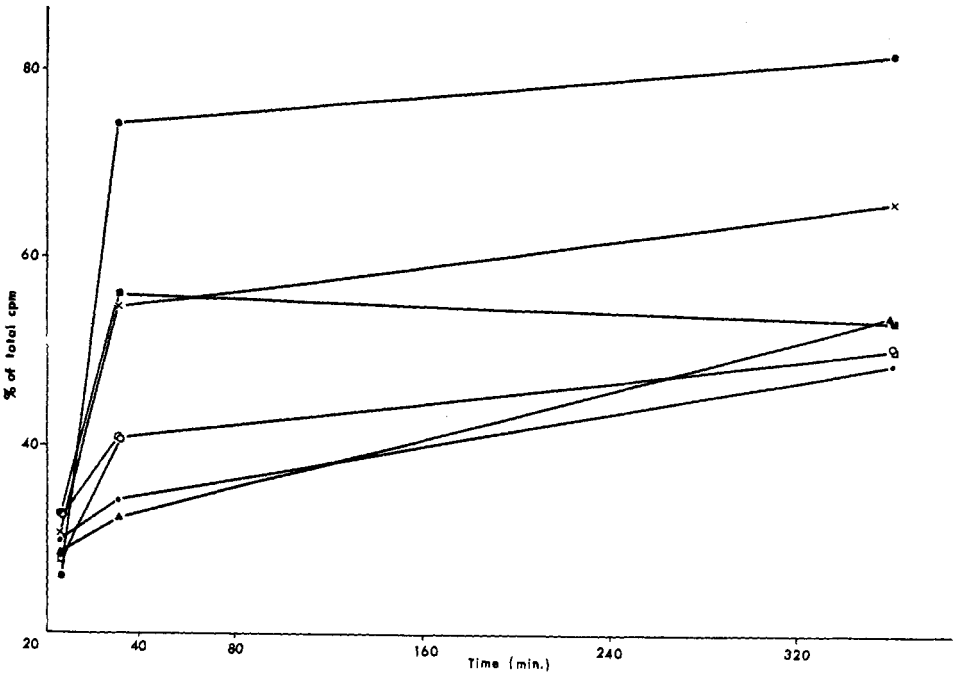

FIGURE 3: Percentages of total lipid radioactivity of isolated cell fractions that is found in phosphonolipid at various times after pulse-labeling cells with chimyl-*t* alcohol. The symbols are the same as used in Figure 2.

fraction, but appreciable activity was also detected in the cilia and the pellicle fractions (Table IV). As we observed earlier with whole cell homogenates (Thompson, 1969), the phosphonolipids were largely spared in these cell fractions, while the enzymes showed a much stronger affinity for phosphatidylcholine and phosphatidylethanolamine. The presence of degradative enzymes in surface membranes could play a role in determining the lipid distribution.

Although we have not conducted *in vitro* incubations with labeled tetrahymanol, we have observed no loss of ^{14}C from radioactive tetrahymanol incubated *in vivo* with logarithmic cells for several hours. During that time, the triterpenoid is rapidly distributed to the various cell fractions.

Discussion

It has been recognized for some time that not all membranes of a particular cell have the same lipid compositions. Fleischer and Rouser (1965) have reviewed some of the earlier findings which indicated certain general trends, such as the localization of cardiolipin in mitochondrial membranes and sterols in the plasma membrane. Additional data confirming high cholesterol levels in surface membranes have been reported from several laboratories (Ashworth and Green, 1966; Coleman and Finean, 1966; Weinstein *et al.*, 1969). The molar ratio of cholesterol to phospholipid in plasma membranes varies from approximately 0.5 to 1.2, while the ratio for whole cell lipids is usually 0.3 or less.

Our findings show that the distribution of tetrahymanol among the subcellular fractions of logarithmic phase *Tetrahymena* is to some extent similar to that of cholesterol in mammalian cells. Each fraction has its characteristic tetrahymanol content, with the surface membranes of the cell being preferentially enriched with respect to the internal membranes. Thus the membranes of the cilia have over seven times as much tetrahymanol as do the microsomal membranes, when measured against the amount of phospholipid present. Even so, the molar ratio of tetrahymanol to phospholipid in cilia is somewhat lower than the average cholesterol to phospholipid ratio in animal cells. The concentration in the microsomal fraction is well below the minimum levels (generally about 0.1 mole of cholesterol/mole of phospholipid) previously reported for sterols or related compounds in animal cell membranes (Fleischer and Rouser, 1965; Ashworth and Green, 1966).

The quantitative determination of tetrahymanol was possible through the development of the gas chromatographic procedure described above. Other gas chromatographic analyses have been performed on either ethyl ether (Conner *et al.*, 1969; Conner and Mallory, 1969) or hexane (Shorb *et al.*, 1965) extracts of lipid hydrolysates or on purified fractions from silicic acid columns (Conner and Ungar, 1964). These published procedures do not involve the use of

TABLE IV: Degradation of Palmitate-^{14}C-Labeled Lipids During the Incubation of Isolated Cell Fractions at 25° for 60 min; Average of Two Experiments.

Cell Fraction	% of Total Lipid Radioactivity Released as Free Fatty Acids
Cilia	12
Ciliary supernatant	3
Pellicle	27
Mitochondria	43[a]
Microsomes	6
Postmicrosomal supernatant	4

[a] Some activity may be caused by contaminating lysosomes.

internal standards and are not sufficiently sensitive for the present work.

While the differences in tetrahymanol concentration are the most pronounced we have detected, there is also, as reported earlier (Nozawa and Thompson, 1971a), a marked variation in phosphonolipid content from one membrane type to another. Again the highest level is found in ciliary membranes, where phosphonolipids account for almost 70% of the lipid phosphorus *vs.* 33% in microsomes.

New data presented above reveal that alkyl glyceryl ethers, existing as components of phospholipids, are likewise nonuniformly distributed within the *Tetrahymena* cell, with the highest enrichments again being found in surface membranes. The evidence implies that in all cell fractions, a high proportion of the glyceryl ether residues exist in the same molecules containing 2-aminoethylphosphonate. We previously showed that these two constituents usually occur together in lipid extracts of the whole cells (Thompson, 1967), and the association has been confirmed by analysis of the purified phosphonolipid (Berger and Hanahan, 1971).

These findings and others relating to localized differences in cellular lipid composition pose a number of questions concerning membrane metabolism and function. One of the most challenging tasks is to discover the cellular mechanisms by which the enrichment of specific lipids in certain membrane systems is achieved. It is generally agreed that most of the enzymes responsible for lipid biosynthesis are located in the endoplasmic reticulum (microsomal fraction) or the cytoplasm of the cell (Jungalwala and Dawson, 1970). Therefore, it is not surprising to find that these two cellular compartments assume the highest initial specific radioactivity after *Tetrahymena* are fed labeled lipid precursors (Nozawa and Thompson, 1971b). The problem is to determine how the cell accomplishes the apparently selective transfer of particular lipid species to sites remote from their origin.

There are two obvious mechanisms by which this localized lipid enrichment might be attained. There may be a degree of specificity associated with the system involved in transporting lipids from the biosynthetic sites to their membrane destinations. Alternatively, at the site of insertion into the membrane there may be a selection of the lipid species necessary for the proper functioning of that membrane. Although the evidence presented in this paper does not allow us to distinguish clearly between these two possibilities, it does provide indirect support for the latter.

The data from the time-course isotope experiments show that the initial patterns of labeling for all membrane fractions are similar. This indicates that the distribution system for lipids conveys the same assortment to all parts of the cell. It is only with the passage of considerable time that the radioactivity patterns begin to differ markedly, finally equilibrating at a point reflecting in each case the quantitative distribution of lipids in that membrane class.

The means by which the surface membranes could select the glyceryl ether containing phosphonolipids and tetrahymanol while rejecting the other phospholipids is not understood. However, the nature of the lipids that accumulate may provide a clue, since they are all remarkably resistant to the endogenous lipolytic enzymes. At first thought, a correlation between lipid composition and enzyme distribution would seem meaningless, since the highest lipolytic activity is in the mitochondrial fraction, which does not have a high percentage of the stable lipids. However, the observed *in vitro* activity may be quite different in rate from that expressed *in vivo*. Perhaps the only pertinent finding is that the surface membranes do have associated lipolytic enzymes.

Even assuming similar *in vitro* and *in vivo* degradation rates, consideration of the findings in the light of our previous results offers some hope of resolving the question. The mitochondria, while capable of the most active lipolysis, exist within the metabolically most active compartment of the cell, as shown by the short time required for them to become highly labeled from added radioactive precursors (Nozawa and Thompson, 1971b). Similar results are observed with nongrowing cells, indicating an active exchange of lipids, perhaps of the type described by Wirtz and Zilversmit (1968). Thus lipid degradation at the rate we observed might have little effect on the composition of an organelle shown to equilibrate its lipids with the adjacent endoplasmic reticulum in 1 hr or less.

On the other hand, the cilia, while only moderately active in phospholipid degradation, are metabolically remote from the site of lipid synthesis, as exemplified by the 6-hr period needed for lipid equilibration. It is conceivable that lipolytic activity in such an isolated membrane could be entrained with the slow lipid addition and exchange processes in such a way as to produce the observed lipid enrichments.

Much additional work is needed to establish a biochemical basis for the unusual lipid distribution within *Tetrahymena*. Equally important is the need to examine the physiological significance of such localized lipid enrichments. There is considerable evidence that the concentration of another triterpene alcohol, cholesterol, is important in determining a membrane's physical characteristics (Chapman, 1969) and permeability properties (McElhaney *et al.*, 1970; Graham and Green, 1970). It will be important to determine if the permeability properties of *Tetrahymena* membranes are likewise affected by their tetrahymanol content.

References

Ashworth, L. A. E., and Green, C. (1966), *Science 151*, 210.

Ball, D. L., Harris, W. C., and Habgood, H. W. (1968), *Anal. Chem. 40*, 129.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Berger, H., and Hanahan, D. J. (1971), *Biochim. Biophys. Acta 238*, 584.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911.

Chapman, D. (1969), *Lipids 4*, 251.

Coleman, R., and Finean, J. B. (1966), *Biochim. Biophys. Acta 125*, 197.

Conner, R. L., and Mallory, F. B. (1969), *Deut. Akad. Wiss. Berlin Vortr. Schr.*, 5.

Conner, R. L., Mallory, F. B., Landrey, J. R., and Iyengar, C. W. L. (1969), *J. Biol. Chem. 244*, 2325.

Conner, R. L., and Ungar, F. (1964), *Exp. Cell Res. 36*, 134.

Fleischer, S., and Rouser, G. (1965), *J. Amer. Oil Chem. Soc. 42*, 588.

Graham, J. M., and Green, C. (1970), *Eur. J. Biochem. 12*, 58.

Hamburger, K., and Zeuthen, E. (1957), *Exp. Cell Res. 13*, 443.

Hanahan, D. J., and Watts, R. (1961), *J. Biol. Chem. 236*, PC 59.

Ikekawa, N. (1969), *Methods Enzymol. 15*, 200.

Jonah, M., and Erwin, J. A. (1971), *Biochim. Biophys. Acta 231*, 80.

Jungalwala, F. B., and Dawson, R. M. C. (1970), *Biochem. J. 117*, 481.

Kennedy, K. E., and Thompson, G. A., Jr. (1970), *Science 168*, 989.
Marinetti, G. V. (1962), *J. Lipid Res. 3*, 1.
McElhaney, R. N., de Gier, J., and van Deenen, L. L. M. (1970), *Biochim. Biophys. Acta 219*, 245.
Nozawa, Y., and Thompson, G. A., Jr. (1971a), *J. Cell Biol. 49*, 712.
Nozawa, Y., and Thompson, G. A., Jr. (1971b), *J. Cell Biol. 49*, 722.
Shorb, M. S., Dunlap, B. E., and Pollard, W. O. (1965), *Proc. Soc. Exp. Biol. Med. 118*, 1140.
Smith, J. D., Snyder, W. R., and Law, J. H. (1970), *Biochem. Biophys. Res. Commun. 36*, 1163.
Thompson, G. A., Jr. (1965), *J. Biol. Chem. 240*, 1912.
Thompson, G. A., Jr. (1967), *Biochemistry 6*, 2015.
Thompson, G. A., Jr. (1969), *Biochim. Biophys. Acta 176*, 330.
Thompson, G. A., Jr., and Kapoulas, V. M. (1969), *Methods Enzymol. 14*, 668.
Weinstein, D. B., Marsh, J. B., Glick, M. C., and Warren, L. (1969), *J. Biol. Chem. 244*, 4103.
Wirtz, K. W. A., and Zilversmit, D. B. (1968), *J. Biol. Chem. 243*, 3596.

A Membrane-Bound Phospholipase A1 Purified from *Escherichia coli**

Carl J. Scandella† and Arthur Kornberg‡

ABSTRACT: Phospholipase A1 bound tightly in the cell membrane hydrolyzes membrane phospholipids following heat treatment, lysis, or aging (48 hr at 0°) of *Escherichia coli* cells. This enzyme may be responsible for phospholipid breakdown and for changes in membrane integrity which have been observed following phage infection, the addition of antibody and complement, or colicin action. We purified the enzyme approximately 5000-fold to near homogeneity by solubilization with sodium dodecyl sulfate (SDS)–butanol, isoelectric precipitation, acetone fractionation, and SDS–acrylamide gel electrophoresis. The enzyme is stable in 3% SDS and tends to aggregate in the absence of detergent. Neither detergent nor the lipids which copurify with the enzyme are necessary for activity. The enzyme hydrolyzes the 1-acyl chain of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol (PG), and diphosphatidylglycerol at comparable rates. The K_m for PG is 3×10^{-7} M. Hydrolysis of L-phosphatidylcholine but not D-phosphatidylcholine or triglyceride identifies the enzyme as phospholipase A1 and distinguishes it from known lipases.

Our work on phospholipids and phospholipase activity in *Escherichia coli* was prompted by an interest in knowing how macromolecules such as proteins and DNA gain passage through the membrane. Phage gene products appear to be necessary in order to allow lysozyme to pass through the membrane of the infected cell (Mukai *et al.*, 1967; Harris *et al.*, 1967) and may be responsible for the dissolution of the membrane during the process of phage infection (Cota-Robles and Coffman, 1964).

We examined lipid extracts from phage-infected cells, hoping to find a chemical basis for this structural alteration. We found that extensive phospholipid hydrolysis accompanies phage-induced lysis. However, sonication and osmotic lysis of uninfected cells also produce this effect. We were thus led to recognize the presence of phospholipase A, an enzyme previously sought in *Escherichia coli* (Proulx and van Deenen, 1967). Recent reports that colicins, human serum, and several phages elicit phospholipase A action in *E. coli* focus additional interest on this enzyme (Cavard *et al.*, 1968; Barbu and Lux, 1969; Cronan and Wulff, 1969; Josslin, 1970; Cohen *et al.*, 1970; Reader and Siminovitch, 1971). While our work on the purification of the enzyme was in progress, Okuyama and Nojima (1969) and Fung and Proulx (1969) reported the presence of phospholipase A in *E. coli*.

Interest in the *E. coli* phospholipase extends beyond its physiologic function because the enzyme offers an opportunity to study membrane structure at the molecular level. The enzyme is bound to the membrane, yet it can be released in an active form by detergents. The enzyme is remarkably stable in the presence of sodium dodecyl sulfate[1] (SDS) and some organic solvents. These attributes together with a rapid assay for enzyme activity enabled us to work out a procedure for purifying this insoluble protein approximately 5000-fold to a nearly homogeneous state. This report describes how the enzyme is purified and some of the interesting properties and specificity of action of the enzyme in the isolated state.

Materials and Methods

E. coli B, grown by the Grain Processing Corp. of

* From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305. *Received July 9, 1971.* Supported by grants from the National Science Foundation and U. S. Public Health Service. Taken in part from a thesis submitted by C. J. S. to Stanford University.

† National Science Foundation Predoctoral Fellow, 1966–1971. Present address: Department of Chemistry, Stanford University.

‡ To whom to address correspondence.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DGP, diphosphatidylglycerol; lysoPG, monoacylglycerophosphorylglycerol; lysoPE, monoacylglycerophosphorylethanolamine; ATP, adenosine 5′-triphosphate; 1 munit = 10^{-3} unit. The reader is referred to Lennarz (1970) for a discussion of phospholipase nomenclature.