Studies of Membrane Formation in *Tetrahymena pyriformis*. I. Rates of Phospholipid Biosynthesis*

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With the Technical Assistance of Pearl Woo

ABSTRACT: Characterization of the principal structural lipids of *Tetrahymena pyriformis* W discloses a unique pattern. Lecithin of the classical type is replaced to a significant degree by the glyceryl ether analog, and the lipid resembling phosphatidylethanolamine also contains considerable amounts of bound glyceryl ethers as well as 2-aminoethylphosphonic acid. The formation of these lipids from a number of radioactive precursors has been studied. The most active precursors examined are palmitic acid, acetic acid, and chimyl alcohol. It is entirely feasible to use these compounds in labeling lipids synthesized during a brief period in the cell cycle. The effects of several experimental variables are assessed in preparation for an evaluation of membrane interrelationships within the cell. The action of a potent system of lipolytic enzymes is measured.

Biologists generally agree that a more thorough knowledge of the processes governing biological membrane formation would fill an important void in our understanding of the cell. In particular, metabolic interrelationships between different membranes within the cell must be essential in controlling growth and cell division. Because of the multitude of membrane types in each cell and the variety of cell types in most tissues, unequivocal experimental results pertaining to possible interrelationships have been difficult to obtain. Our efforts to single out a system simple enough to be useful have led us to examine cultures of the protozoan *Tetrahymena pyriformis*.

This organism has certain obvious advantages for the study of membrane metabolism. It grows rapidly, and all cells can be assumed to be identical in their metabolism, particularly so when the cells are dividing synchronously. Although it is one of the most primitive animal organisms, its intracellular organelles and membrane systems resemble those found in mammalian cells (Elliott, 1963). Being unicellular, it can be subjected to precisely timed pulse-labeling studies with radioactive precursors.

It was upon this last property that we wished to base a series of experiments involving precursors of membrane lipids. It would seem that a correlation of the rates of structural lipid synthesis in various classes of cellular membranes might shed some light on their origin and test the popular hypothesis (Robertson, 1964) that certain membranes arise through modification of morphologically different ones.

Unfortunately, the literature provides little detailed information of any type concerning the metabolism of *Tetrahymena* structural lipids (Erwin and Bloch, 1963; Liang and Rosenberg, 1966). In this initial communication, we present data which describe the formation of lipids from various precursors and suggest that *Tetrahymena* is in some ways uniquely suited for the studies proposed above. In interpreting our results, we have accepted the widely held (Ansell and Hawthorne, 1964; Dawson, 1966) but not rigorously proven view that essentially all cellular phospholipids exist as structural components of membranes.

Experimental Methods

Culture Conditions. All analyses have employed *T. pyriformis* W, a gift from Dr. A. M. Elliott, University of Michigan. Stock cultures were maintained in tubes of 2% proteose peptone. Logarithmic phase cultures were routinely grown under the following conditions. Approximately 3×10^5 cells were added from a stock culture to a 500-ml erlenmeyer flask containing 200 ml of a medium composed of 2% proteose peptone, 0.5% dextrose, 0.2% yeast extract, and 2 ml of 9 mM Fe^{3+}–EDTA complex (Conner and Cline, 1964). Aeration was provided by a 4.5-cm long magnetic stirring bar turning at 200 rpm, and the temperature was constant at 22°. In some cases the preparations were scaled up in size and aerated by shaking.

Culture growth was monitored by diluting formaldehyde-killed organisms with 0.1 M KCl and counting cell density in a hemocytometer. At the desired time the contents of the culture flask were rapidly (1–2 min)

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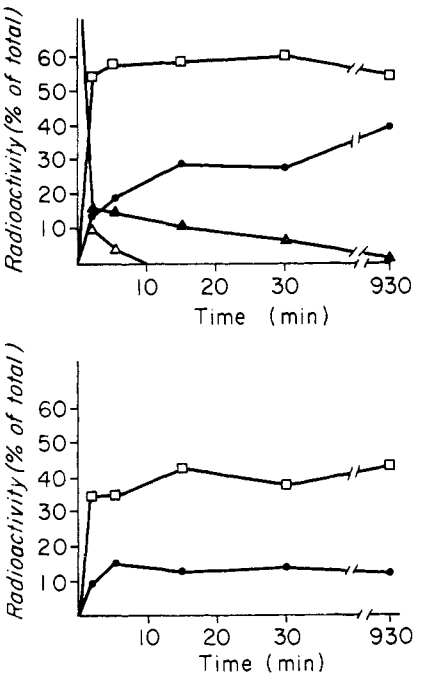


FIGURE 1: Incorporation into lipids and phospholipids of 0.002 μmole of [^{14}C]palmitate/ml added to a log-phase culture at 0 min. Aliquots of the culture were removed after the time periods indicated and pipetted directly into extracting solvents without separation of cells from medium. Data are expressed as the per cent of total lipid radioactivity recovered from each aliquot. (A) (top) (□) Total phospholipid. (●) Triglycerides. (△) Diglycerides. (▲) Fatty acids. (B) (bottom) (□) Ethanolamine phospholipids. (●) Choline phospholipids.

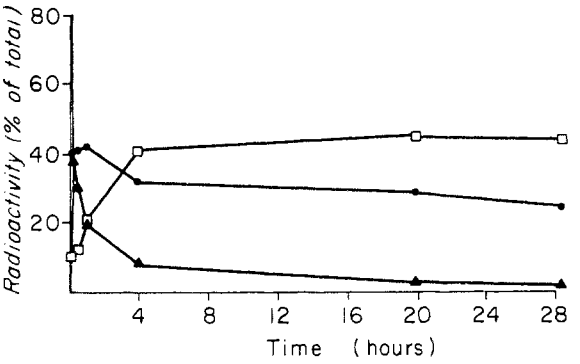


FIGURE 2: Incorporation into phospholipids of 0.01 mμmole of [^{3}H]chimyl alcohol/ml added to a log-phase culture at zero time. Aliquots of the culture were removed after the time periods indicated and pipetted directly into extracting solvents without separation of cells from medium. Data are expressed as the per cent of total lipid radioactivity recovered from each aliquot. (□) Ethanolamine phospholipids. (●) Choline phospholipids. (▲) Glyceryl ethers.

cooled to 0–4° in a Dry Ice–acetone bath and centrifuged at 1000*g* or, in some cases, at lower speeds, for 15 min in a refrigerated centrifuge. After resuspending in cold distilled water and recentrifuging, the cells were taken up in a minimal volume of cold 0.1 M KCl. Lipids were extracted by the method of Bligh and Dyer (1959). The extraction of lipid by the method of Folch *et al.* (1957) was occasionally used with comparable results. In experiments where cells were to be grown at temperatures other than 22°, the culture was cooled or warmed quickly and incubated with shaking or stirring in a constant-temperature bath.

Chemical Methods. Thin layer chromatography (tlc) of the lipids was carried out using the following solvents in volume proportions: petroleum ether (bp 30–60°)–ethyl ether–acetic acid (70:30:1) for total lipids, petroleum ether–ethyl ether–acetic acid (30:70:1) for glyceryl ethers, and chloroform–methanol–water (95:35:4) for phospholipids. Radioactivity measurements of ^{14}C were accomplished by scraping the desired area of the thin layer plate into a scintillation vial containing 3% Cabosil (Cabot Corp.) in a toluene base scintillation fluid. Complete recovery of ^{3}H from plates could be obtained only by moistening the scraped silica gel with 10 drops of Hyamine hydroxide (Packard Instrument Co.) solution to effect release of lipids when scintillation fluid was subsequently added. Cabosil was not used with ^{3}H.

Column chromatography on silicic acid was performed as previously described (Thompson and Hanahan, 1963) except that chloroform–methanol (9:1, v/v) was passed through the column before eluting with chloroform–methanol (6:1). Fractions were identified by tlc, infrared spectrophotometry, and the following quantitative methods. Fatty acid esters were measured by infrared spectrophotometry (Freeman, 1957), phosphorus by the method of Bartlett (1959) modified by digestion with perchloric acid according to Marinetti (1962), and nitrogen by the method of Lang (1958) as modified by M. Wells. Total lipids and glyceryl ethers, after chromatographic purification (Thompson, 1965), were quantified by weighing in tared flasks. Plasmalogens were determined by the method of Gottfried and Rapport (1962) or by analysis of $LiAlH_4$ reduction products (Thompson, 1965). Water-soluble lipid constituents were released by hydrolysis in refluxing 6 N HCl for 3 hr, or, in the case of AEP,[1] sealed-tube hydrolysis in 6 N HCl at 110° for 24 hr. The latter procedure was shown to hydrolyze the phosphorus of egg lecithin completely to inorganic phosphate while causing no degradation of AEP (Sigma). Hydrolysis products were chromatographed on paper using the systems: H_2O-saturated phenol (Hori *et al.*, 1964), 1-butanol–acetic acid–H_2O (4:2:1) (Hori *et al.*, 1964), ethyl acetate–pyridine–H_2O (25:10:25) (Sastry and Kates, 1964) for glycerol

[1] Abbreviation used: AEP, 2-aminoethylphosphonic acid.

and inositol only, and 1-propanol–H_2O (80:20) for choline only. Further identification of AEP, phosphorylethanolamine, and phosphorylcholine was made by comparison with authentic standards on columns of Dowex 50 ion-exchange resin (V. M. Kapoulas, unpublished observations). A neutral lipid tentatively identified as tetrahymanol by its chromatographic behavior and infrared spectrum was present in all cultures. It was resistant to acid and base hydrolysis. Purification by recrystallization from hexane and aqueous ethanol yielded a white solid, mp 297–298° (uncor), lit. (Tsuda *et al.*, 1965) mp 295–296°.

Labeling Experiments. [1-^{14}C]Palmitic acid (10 mc/mmole), [U-^{14}C]glucose (11.4 mc/mmole), sodium [1-^{14}C]acetate (52.5 mc/mmole), [1,2-^{14}C]ethanolamine (4.3 mc/mmole), and [1,3-^{14}C]glycerol (12 mc/mmole) were purchased from New England Nuclear Corp. [6-^{14}C]Glucose (25 mc/mmole) was obtained from Nuclear-Chicago Corp. The preparation of [^{3}H]-chimyl alcohol has been described previously (Thompson, 1965). All isotopes were measured on a Packard Tri-Carb scintillation spectrometer with efficiencies of 49% for ^{3}H and 89% for ^{14}C. Quenching was determined by external standardization.

Water-soluble substrates were added to cultures in aqueous solution. Palmitic acid, chimyl alcohol, and phospholipids were added in 2–4 drops of ethanol. If the latter substrates were not injected below the surface of a rapidly stirred medium, a certain percentage would sometimes adhere to the vessel wall and become unavailable to the cells.

Analyses of the short-term incorporation experiments, such as those illustrated in Figures 1 and 2, were made by pipetting aliquots of medium plus cells directly into the Bligh-Dyer extracting solvents.

Cell homogenates were prepared with a hand-operated Potter-Elvehjem homogenizer. The small percentage of cells remaining unbroken was not motile. Homogenates with or without potential inhibitors were incubated with gentle shaking at room temperature for 1–3 hr before lipid extraction.

Results

Nature of the Lipids. The growth rate of *Tetrahymena* cultures is strongly dependent upon the composition of the medium and the degree of aeration. When grown under our standard conditions as described in the Methods section, cultures enter the log phase of growth after 12 hr and reach the stationary phase (1.8–2.0 $\times$ 10^6 cells/ml) after 50 hr. During the log phase the lipid composition consists of 0.2 μmole of phospholipid and 0.02 μmole of tetrahymanol/10^6 cells. Traces of ubiquinone$_8$ and other hydrocarbons, free fatty acids, triglycerides, and diacyl glyceryl ethers also occur. As the culture approaches the stationary phase, increasing amounts of triglycerides and fatty acids are formed. In time the accumulation of triglycerides may approach on a weight basis the phospholipid content.

When grown on a less enriched medium, such as unsupplemented 2% proteose peptone, or with less aeration, the cells enter the stationary phase at a much lower cell density and accumulate more triglycerides. However, during logarithmic growth the lipid composition is little different from that described above.

TABLE I: Separation of Phospholipids from Cells of *T. pyriformis* on Columns of Silicic Acid.[a]

Fraction	Eluent[b]	% Total Phosphorus (av of 5 expt)	Water-Soluble Hydrolysis Products
I	CM 9:1	9	Not examined
II	CM 6:1	60	Glycerol, ethanolamine, P_i, and AEP
III	EM 3:2	11	Inositol, glycerol, P_i, ethanolamine, and AEP
IV	CM 1:1	20	Glycerol, P_i, choline, traces of ethanolamine, and AEP

[a] After removing neutral lipids by elution with chloroform, 4-ml fractions were collected and pooled after identification by thin layer chromatography. Recoveries of phospholipid phosphorus ranged from 90 to 101%. [b] C, chloroform; M, methanol; E, ethyl acetate.

The phospholipid distribution is indicated in Table I. The principal components are "phosphatidylethanolamine" and "phosphatidylcholine," which account for 60 and 20 mole %, respectively, of the lipid phosphorus. In addition, there are two small fractions, one of which (I) resembles chromatographically phosphatidic acid and cardiolipin, and the other (III) consisting mainly of phosphatidylinositol plus some "lysophosphatidylethanolamine." A full characterization of the minor fractions will be presented in conjunction with another study dealing with glyceryl ether metabolism (V. M. Kapoulas, unpublished observations). Because the two principal fractions incorporate the majority of radioactivity with all precursors tested, their composition will be described in more detail here.

It is clear from the data in Table II that each of the major fractions is a mixture of more than one molecular type. The speculations of Hack *et al.* (1962) and Taketomi (1961) that glyceryl ether lipids are present have been confirmed. The glyceryl ethers isolated from both fractions are almost entirely chimyl alcohol. In fact, chimyl alcohol accounts for greater than 98 mole % of the glyceryl ethers obtained from the total phospholipid fraction. Fraction II is rich in the derivatives of AEP. Our failure to find indications of sphingosine bases by infrared analysis or hydrolytic procedures

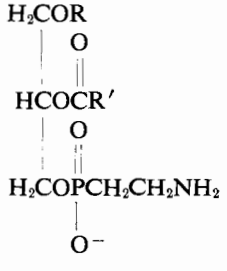
TABLE II: Composition of the Two Major Phospholipid Fractions from *Tetrahymena* Cells Harvested near the End of the Log Phase of Growth.

Fraction	II	IV
% phosphorus (% of total weight)	3.45	3.40
Nitrogen:phosphorus[a]	1.13	1.01
Fatty acid ester:phosphorus[a]	1.44	1.04
Glyceryl ether:phosphorus[a]	0.22	0.62
Phosphonic acid:total phosphorus[a]	0.35	0.08

[a] All ratios given on a molar basis.

agreed with the evidence reported by Liang and Rosenberg (1966) that AEP is present in fraction II as the diacyl analog of phosphatidylethanolamine. However, when fraction II was hydrolyzed in ethanolic KOH as employed by Hanahan *et al.* (1963) to separate derivatives of glyceryl ether phosphatides from diacylphosphatides, an unexpected pattern of association was detected. Whereas the diacylphosphatides (yielding water-soluble phosphorus) contained only 12–20 mole % AEP, glyceryl ether analogs (remaining in the organic solvent phase) contained 70–80 mole % AEP.

The major ether-soluble products of this ethanolic KOH treatment were fatty acids and a phosphorus-containing, ninhydrin-positive compound resembling in its chromatographic behavior lysophosphatidylethanolamine. Analysis of this latter material after purification on a column of silicic acid established the following properties. The nitrogen to phosphorus ratio was 1.08. Of the total phosphorus, 98% existed as a phosphonic acid. The absence of an absorption band in the infrared spectrum at 5.75 μ indicates that no acyl esters were present. Acetolysis and saponification of the compound yielded, besides a certain amount of the unchanged starting material, only glyceryl ethers as ether-soluble products. Thus it appears certain that the parent lipid is quite unlike the sphingosine-based phosphonic acid derivative isolated from other organisms (Hori *et al.*, 1966) and may be represented by formula I.



I

At least a portion of the phosphonic acid indicated in fraction IV is the result of contamination by lysophosphatidylethanolamine. The phosphonic acid analog of phosphorylcholine has not been detected.

There appear to be significant variations in both glyceryl ether content and AEP content as the cultures age. AEP increases in fraction II from approximately 20 mole % in the middle log-phase cells to 44 mole % in stationary-phase cultures. The glyceryl ether levels in fractions II and IV increase from 15 to 20 and from 50 to 70 mole %, respectively, as the cultures develop from logarithmic growth to the stationary phase.

Hydrolytic degradation of fraction IV gives rise to small amounts of a compound which resembles glyceryl ethers in its infrared spectrum but has different chromatographic properties. This component may account in part for the unexpectedly low ester:phosphorus ratio. A somewhat similar compound is found in fraction II hydrolysates. Efforts to characterize these materials are underway. Contrary to the report of Taketomi (1961), neither the total lipid extract nor any fractions were found to contain plasmalogen, although column chromatography at 4° was employed to prevent possible degradation.

Incorporation of Precursors by Cultures in the Logarithmic Growth Phase. The uptake and incorporation into lipids of a number of potential precursors were tested in an effort to find compounds which would rapidly be transformed into structural lipids. Growth of *Tetrahymena* in medium containing [^{14}C]glucose resulted in less than 1% incorporation of tracer into lipid either in short-term (0.12 μmole of [6-^{14}C]glucose/ml for 4 hr) or long-term (0.018 μmole of [U-^{14}C]-glucose/ml for 3 days) experiments. Likewise, only traces of radioactivity were found in lipids after growth for 2 hr in 0.006 μmole of [1,3-^{14}C]glycerol/ml. The incorporation of [1,2-^{14}C]ethanolamine was somewhat more active, with approximately 1% of the added material (0.26 μmole/ml) being converted to lipid after 24 hr. In this case 69% of the radioactivity was found in "ethanolamine" phospholipids and 24% in choline phospholipids. Acid hydrolysis of the former fraction released radioactive ethanolamine but unlabeled AEP. Such evidence does not support the proposal of Segal (1965) that AEP arises from lipid-bound ethanolamine.

In sharp contrast to the compounds described above, [1-^{14}C]acetate was readily incorporated into *Tetrahymena* lipids. [^{14}C]Acetate fed at a concentration of 0.004 μmole/ml was converted to lipids at a constant rate of 0.3%/min for 2 hr. Addition of a 100-fold excess of unlabeled acetate during this period led to an immediate sharp decrease in the incorporation of radioactivity. In longer term experiments (0.0075 μmole) of acetate/ml for 4 days and 0.002 μmole of acetate/ml for 1 day), 50 and 65%, respectively, of the fed radioactivity was recovered in lipids. The intact lipids were fairly uniformly labeled on a molar basis. Rather surprisingly, 2 N HCl hydrolysis of the neutral lipids or acetolysis and saponification of the phospholipids demonstrated that acetate had been converted almost

solely to ether-soluble products, *i.e.*, fatty acids, glyceryl ethers, and tetrahymanol.

Even more rapidly incorporated into lipids was [1-^{14}C]palmitic acid. When the substrate was added to late log-phase cultures at a concentration of 0.002 μmole/ml, incorporation was 85% complete within 5 min. As indicated by Figure 1A, most of the radioactivity entered phospholipids. Figure 1B shows that the phospholipid radioactivity is at all time intervals principally in the "ethanolamine" and choline fractions. Several similar experiments have shown that minor differences in cell density or culture conditions do not appreciably alter the rate of palmitate incorporation. The distribution pattern of radioactivity has been confirmed by more than ten separate labeling experiments with [^{14}C]palmitate. Once incorporation is complete, the percentage of radioactivity in each phospholipid fraction remains essentially unchanged for at least 24 hr (six to eight cell doublings) after substrate administration.

Equilibration of phospholipid fatty acids with a free fatty acid or glyceride pool appears to be minimal. When a dose of radioactive palmitate was followed after 5 min by a 1000-fold excess of unlabeled palmitate, no diminution of phospholipid radioactivity could be detected in aliquots analyzed over the course of an additional 60-min period. Although the size of the intracellular pool of free fatty acids was not determined, uptake was evident from the rapid increase in the triglyceride fraction.

While the percentage distribution of label among the phospholipids does not vary, the division between total phospholipids and total neutral lipids depends upon the age of the culture. Early log-phase cells incorporate [^{14}C]palmitate into phospholipids exclusively, but cells in the stationary phase fix less than 30% of the isotope into phospholipids, the remainder entering glycerides and other nonphosphorus-containing lipids.

A limited amount of the [^{14}C]palmitate is converted to the glyceryl ether moiety of the phospholipids. The specific radioactivity of the glyceryl ethers was determined to be only 25–30% of that of intact "ethanolamine" or choline lipids, even 24 hr after administration of substrate.

The final precursor tested, [^{3}H]chimyl alcohol, also entered the phospholipids rapidly. In an experiment involving growth in a medium containing 0.01 mμmole of [^{3}H]chimyl alcohol/ml, the data presented in Figure 2 were obtained. The radioactivity not plotted was determined to be predominantly in the mono- and diacyl glyceryl ether fractions. In this particular experiment, the aliquots (cells plus medium) extracted each contained only 60% of the expected radioactivity owing to irreversible adsorption of a portion of the substrate to the culture flask walls, but similar distributions are observed in cases where the recovery of radioactivity is 90% of that administered. The curve for the uptake of [^{3}H]chimyl alcohol into choline lipids resembles that found for the incorporation of [^{14}C]-palmitate. This is not true of the uptake by the "ethanolamine" lipids. Here an appreciable lag period preceded the appearance of significant levels of radioactivity. This unusual lag in incorporation of chimyl alcohol by "ethanolamine" lipids is reproducible. Since glyceryl ethers of this fraction are predominantly bound to AEP, the slower rate of formation might imply a more involved biosynthetic pathway. The degradation of [^{3}H]glyceryl ethers to fatty acids, as determined by acetolysis and saponification, was almost negligible, ranging from 3 (after 30 min) to 8% (after 28.5 hr).

In an effort to determine if phospholipids can be incorporated intact into membranes by *Tetrahymena*, [^{14}C]palmitate-labeled "phosphatidylethanolamine" was isolated and added back to a culture at a concentration of approximately 0.1 μmole/ml. Aliquots analyzed 10 and 60 min afterwards both showed a distribution of label among the lipids similar to that found after feeding [^{14}C]palmitate itself. Thus the added phospholipid must have been degraded during the process of assimilation.

Effects of Culture Conditions on Precursor Uptake. Physiological experiments have shown that pronounced alterations in the development of certain subcellular structures, including membrane systems, can be induced in *Tetrahymena* by changing the culture conditions (Elliott and Clemmons, 1966; Scherbaum and Zeuthen, 1954; Padilla *et al.*, 1966). Since this possibility of promoting the selective formation of specific membranes looms large in our proposed studies, the incorporation of one lipid precursor, [^{14}C]palmitate, was measured in cultures of *Tetrahymena* grown under a variety of potentially useful conditions. The results are briefly outlined below.

Because high- and low-temperature shock techniques have been very successful in inducing synchronous division of *Tetrahymena*, we wished to compare the rates of lipid metabolism at various temperatures. At lower temperatures the incorporation of [^{14}C]palmitate was similar to that found in the routine 22° cultures except that the uptake of radioactivity proceeded at a slower rate. In three comparable experiments the incorporation into phospholipids after 5 min was 51% at 15° and 44% at 7° as compared to 58% at 22°. Below 4° incorporation was negligible. At 34°, the temperature employed by Scherbaum and Zeuthen (1954) for producing synchronous cultures, a quite abnormal pattern was observed. When palmitate was added to cells preincubated at 34° for 20 min, there was an initial rapid appearance of radioactivity in triglycerides, amounting to 40% of the total after 2 min. Only 25% of the radioactivity was found in phospholipids at this time. After 10 min the figures for triglycerides and phospholipids, respectively, were 32 and 44%. All aliquots taken (2, 5, and 10 min) contained an unknown nonphosphorus-containing compound appearing on thin layer plates between "ethanolamine" phospholipids and choline phospholipids. Approximately 10% of the total radioactivity accompanied this spot.

Significant levels of radioactivity in this unknown material were also observed under other experimental

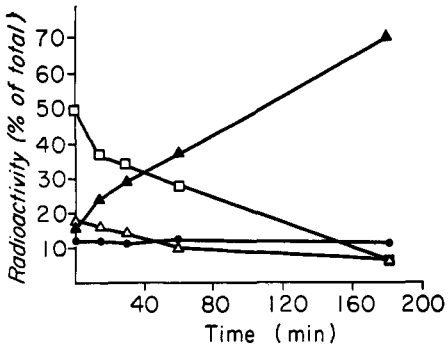


FIGURE 3: Degradation of lipids in a homogenate of log-phase cells grown for 30 min in a medium containing 3×10^{-4} μmole of [^{14}C]palmitate/ml. Homogenate prepared at 0° and raised to 23° at 0 min. (□) Ethanolamine phospholipids. (△) Choline phospholipids. (▲) Fatty acids. (●) Triglycerides.

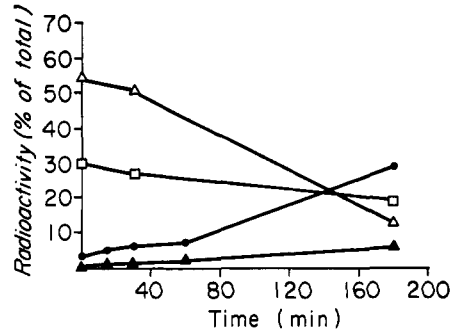


FIGURE 4: Degradation of lipids in a homogenate of log-phase cells grown for 60 min in a medium containing 0.02 mμmole of [^{3}H]chimyl alcohol/ml. Homogenate prepared at 0° and raised to 23° at 0 min. (□) Ethanolamine phospholipids. (△) Choline phospholipids. (●) Glyceryl ethers. (▲) Fatty acids.

conditions. The unknown compound was a prominent component in cultures maintained for 2 days in the inorganic medium of Hamburger and Zeuthen (1957). Under these conditions, where no external source of organic nutrients is available, cell division ceases. The esterification of fed [^{14}C]palmitate is almost as rapid as is found with dividing cultures, but the incorporation pattern is more akin to that described above for cultures grown at 34°. Radioactivity in the unknown material amounts to as much as 19% of the total after 30 min and 15% after 2 hr. A final instance where incorporation of palmitate into the unknown component was observed involved cells in the stationary phase of growth. Thus in all cases this particular pattern was found when cell division had ceased.

Action of Lipolytic Enzymes. In the course of these experiments it was discovered that *Tetrahymena* contains a system of lipolytic enzymes which is activated by manipulations not ordinarily expected to injure the cells. Centrifugation of the cells at room temperature or simply allowing cells to settle by slowly cooling the culture flask to 4° results in noticeable increases in the concentration of free fatty acids. The release of fatty acids is more pronounced in homogenates of the cells. A very sensitive estimate of lipid degradation may be obtained by analyzing aliquots withdrawn at various times from incubating homogenates of isotopically labeled cells. Thin layer chromatography of lipids from these aliquots provides a visual indication that degradation is proceeding. The spot representing fatty acids becomes more pronounced as degradation proceeds, and a depletion of the phospholipids can be discerned. The curves of radioactivity distribution in Figure 3 were obtained by counting areas scraped from these thin layer plates. The degradation of "ethanolamine" phosphatides is most rapid although choline phosphatides are also degraded. At no time interval examined was there any indication of lysophosphatides, either by radioactivity measurements or visual examination of the plates. The gradual decrease of lipid phosphorus but not chloroform-soluble radioactivity further attested to the degradation of lipids to fatty acids and water-soluble products.

The degradation of [^{3}H]chimyl alcohol labeled lipids was also examined (Figure 4). One particularly notable difference was found in the rates of enzymatic attack. The glyceryl ether labeled "ethanolamine" phospholipids were broken down at a much slower rate than found in [^{14}C]palmitate-labeling experiments. Again this could be an influence of the phosphonate group. Only after the 3-hr incubation could a clearly visible spot corresponding to free glyceryl ethers be detected on thin layer plates. A small amount of radioactivity appeared in the area corresponding to lysolecithin.

Examination of the fatty acids released during the various time intervals by the use of gas–liquid partition chromatography showed that no significant composition changes occurred as degradation proceeded. In all cases the distribution of fatty acids was similar to that described for *Tetrahymena* phospholipids by Erwin and Bloch (1963).

The effective separation of subcellular organelles containing intact membranes was hampered by this lipolytic reaction, which can proceed to a measurable extent during a lengthy centrifugation even when care is taken to maintain the samples at 4° or below. The degradation accompanied cell breakage in all media tested, including buffered 0.25 or 0.7 M sucrose, 0.15 M KCl, or the media employed with *Tetrahymena* by Kobayashi (1965). Consequently, an effort was made to block the degradation by the use of inhibitors. The lipolytic activity, as measured in 0.25 M sucrose homogenates at room temperature, was not appreciably inhibited by 20 mM EDTA, 30 mg/ml of bovine serum albumin, 48 mM citrate, 0.02 mM Cu^{2+}, 0.1 mM Hg^{2+}, 1 mM iodoacetate, 1 mM iodoacetamide, 1 mM diisopropylphosphorofluoridate, 0.45 mM diethyl-*p*-nitrophenyl phosphate (paraoxon), or 20 mM sodium *p*-chloromercuribenzenesulfonate. Sodium desoxy-

cholate (0.24 or 1.6 mg/ml) stimulated degradation. Fluoride at a concentration of 41 mM reduced the release of radioactive fatty acid from prelabeled cells by 20–30%. The use of 0.25 M sucrose 41 mM in sodium fluoride as a disrupting medium and painstaking care to maintain a low temperature during fractionation appear to eliminate the problem of lipolysis.

Discussion

In view of the enticing biological possibilities of *Tetrahymena* as a system for studying membrane interrelations, we have examined the feasibility of pertinent biochemical experiments. The results outlined above indicate that by following the destiny of newly synthesized structural lipids, much can be learned about the origin and fate of lipoprotein membranes.

A cursory examination of the structural lipid composition of *Tetrahymena* leaves an impression of relative simplicity; only one neutral lipid (tetrahymanol) and two major phospholipids (phosphatidylethanolamine and phosphatidylcholine) are present. Closer scrutiny of the principal phospholipid fractions reveals a number of unusual characteristics. The choline lipids are rich in glyceryl ether analogs of lecithin, and the "ethanolamine" fraction is in fact a mixture of four types, combining the acyl and ether moieties with either the ethanolamine base or the recently discovered (Liang and Rosenberg, 1966) 2-aminoethylphosphonic acid. The rate of lipid metabolism is in general extremely rapid. The incorporation of tracer amounts of [^{14}C]-palmitate is essentially complete within 5 min. Under the growth conditions employed, this is less than 3% of the generation time and as such, should amount to a pulse labeling of only that small fraction of membranes being formed during the period of uptake.

The practicability of following these [^{14}C]palmitate-labeled structural lipids to determine the fate of newly formed membranes depends upon the nonexchangebility of the esterified palmitate. The well-known tendency for phospholipid-bound fatty acids to exchange with the fatty acid pool in certain cells (Mulder and Van Deenen, 1965) requires that caution be used in interpreting findings. However, the constancy of the labeling pattern, once established, even in the presence of a large excess of nonlabeled palmitate, suggests that exchange is not quantitatively important in rapidly growing cultures.

Comparable experiments were performed using [^{3}H]chimyl alcohol. As in molluscan tissues (Thompson, 1965), [^{3}H]chimyl alcohol can be incorporated intact into *Tetrahymena* phospholipids. Very little degradation of the precursor occurs. The incorporation into choline phospholipids proceeds at a rate comparable to that observed for [^{14}C]palmitate. However, the incorporation into the "ethanolamine" lipids is unexpectedly slow. Interestingly, the glyceryl ethers of this latter fraction are principally associated with AEP. Rosenberg (1964) has recently shown that the incorporation of $^{32}P_i$ into lipid-bound AEP is less rapid than into diesterified phosphorus.

With respect to the potential problem of fatty acid exchange mentioned above, the ability of *Tetrahymena* to utilize fed glyceryl ethers is a distinct advantage. The likelihood of exchange of this structural component, comprising the glyceryl backbone of the phospholipid molecule as well as one hydrocarbon side chain, is less than might be expected for fatty acids. Thus, comparison of membrane fractions from [^{3}H]-chimyl alcohol labeled cells with equivalent fractions from [^{14}C]palmitate cells would provide a measure of any [^{14}C]fatty acid exchange.

Besides exchange of components of phospholipid molecules, it is possible that the entire molecules might move from one membrane to another. At the present time no information is available concerning such intracellular migration.

A third method available for rapidly labeling cellular lipids involves the feeding of [^{14}C]acetate. The finding that essentially all the radioactivity from lipids of [^{14}C]acetate-fed cells is contained in the nonpolar lipid constituents simplifies the interpretation of resulting data. Any differences in distribution of radioactivity in subcellular organelles from [^{14}C]palmitate and [^{14}C]-acetate experiments would imply that the site of intracellular synthesis depends to some extent upon whether the fatty acids involved are of endogenous or exogenous origin. In the natural state both sources are important.

Among the interesting characteristics of *Tetrahymena* is the potent system of lipolytic enzymes. Frequent references have been made in the literature to inhibiting substances present in *Tetrahymena* extracts. For example, Eichel (1959, 1960) found in *Tetrahymena* extracts aged at 0° a powerful inhibitor of electron transport that was partially inactivated by bovine serum albumin and was mimiced by the products of phospholipase A action. These observations led him to suggest that either fatty acids or lysophosphatides were responsible for the inhibition noted. The data reported here establish the nature of the active compound. In studies of membrane metabolism it is particularly important to avoid even a small degree of lipolysis. Because the products of such a reaction have detergent properties, they could lead to solubilization of some membrane components, thus confusing the results of isotope experiments.

The next phase of this project is to carry out short-term labeling experiments followed by analyses of various purified membrane fractions. A number of satisfactory schemes for the separation of *Tetrahymena* organelles have been reported (Kobayashi, 1965; Suyama, 1966; Lee and Scherbaum, 1965; Gibbons, 1965). Testing and modification of these procedures are now in progress in our laboratories.

The potential of *Tetrahymena* for use in studies of membrane metabolism during rapid growth, nongrowing conditions, synchronized division, senesence, and phagocytosis will hopefully allow the effects of these phenomena upon the cell's economy to be evaluated. The similarity of this cell to cells of higher organisms suggests that the information gained here will be widely applicable.

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