

STUDIES OF MEMBRANE FORMATION IN *TETRAHYMENA PYRIFORMIS*

III. Lipid Incorporation into Various Cellular Membranes of Logarithmic Phase Cultures

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ABSTRACT

When $1\text{-}^{14}\text{C}$ -palmitic acid is used to pulse label logarithmic cultures of *Tetrahymena pyriformis*, radioactivity appears in lipids of the various membrane types at vastly differing rates. The microsomes and postmicrosomal supernatant attain a high specific radioactivity within 1 min, while the membranes enveloping the cilia require several hours to reach the microsomal level. A similar pattern is obtained when the tracer is sodium $1\text{-}^{14}\text{C}$ -acetate or $8,9\text{-}^3\text{H}$ -hexadecyl glycerol. In all fractions the phospholipid incorporates radioactivity from ^{14}C -palmitate much less rapidly than do the other major phospholipids. The patterns of labeling suggest that new lipids are transported from a cytoplasmic site of synthesis to points of membrane fabrication throughout the cell.

INTRODUCTION

One of the most intriguing problems of cell biology today concerns the mechanism whereby protein and lipid subunits are assembled into metabolically active cellular membranes. New findings have seemed to complicate further this little understood process. Until recently, most popular models for membrane biosynthesis have assumed that lipids and proteins are inserted into expanding membranes simultaneously, perhaps in the form of a lipoprotein subunit. However, current observations (1, 2, 3, 4) indicate that under certain conditions these two major membrane constituents may be added to a membrane independently of one another. Other evidence (5, 6, 7, 8) suggests that at least some structural elements of

membranes rapidly exchange with a cytoplasmic pool of membrane precursors.

These new data point up the need to modify and refine existing models for membrane biogenesis. Yet there is still insufficient information to support an hypothesis encompassing the entire process of membrane fabrication. We feel that full understanding of the phenomenon requires a more penetrating analysis than has customarily been made. This more complete analysis should include a comparison of all parts of the cellular membrane system.

The ciliate *Tetrahymena pyriformis* possesses certain characteristics which make it particularly well suited for the study of membrane biogenesis.

We have shown in the accompanying paper (9) that a variety of distinct membrane types can conveniently be isolated. In this paper we describe our initial data regarding the incorporation of lipid precursors into these membranes of logarithmically growing cells. The potential of *Tetrahymena* in studying the control mechanisms used for diversion of membrane precursors to specific organelles will be discussed in the light of these findings.

MATERIALS AND METHODS

The radioisotope labeling experiments utilized 1-¹⁴C-palmitic acid (54 mCi/mmol) and sodium-1-¹⁴C-acetate (56.9 mCi/mmol) from New England Nuclear Corp., Boston, Mass., and ³H-chimyl alcohol (1-O[8,9-³H] hexadecyl glycerol, 1 × 10³ mCi/mmol) prepared as described earlier (10). The palmitic acid and chimyl alcohol were repurified by preparative thin-layer chromatography before use. These latter two substrates were each added in amounts of 1–2 × 10⁶ dpm to 200 ml logarithmic phase (4–6 × 10⁵ cells/ml)¹ cultures of *Tetrahymena pyriformis*, strain E, by dissolving them in 2–4 drops of ethanol and rapidly injecting the solution below the surface of the stirred culture. Sodium-1-¹⁴C-acetate was added in aqueous solution.

In order to achieve a pulse incorporation of ¹⁴C-acetate comparable to the 5-min interval required for complete ¹⁴C-palmitate uptake, the following procedure was followed. 50 μCi of ¹⁴C-acetate in aqueous solution were added to the culture. After an incubation period of 5 min, the cells were centrifuged at 164 g for 5 min at room temperature and resuspended in 200 ml fresh medium containing 5 mg/ml of unlabeled sodium acetate. Then the cells were centrifuged again and resuspended in another 200 ml portion of the acetate-supplemented medium for the remainder of the incubation period.

At the end of the desired time interval, labeled cultures were cooled to 4°C within 1 min with an acetone: Dry Ice bath. The chilled cells were harvested as previously described (9). The washed cells were homogenized and separated into individual subcellular fractions as outlined in the accompanying paper (9). Methods for lipid extraction and analysis are also given in the companion paper (9). Radioactivity was measured with a Packard Model 3310 scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) having a counting efficiency of

¹Incubations planned for 12 hr were begun with younger cultures to insure that they would remain in the logarithmic phase of growth throughout the experiment.

89% for ¹⁴C and 49% for ³H. The culture conditions for *Tetrahymena* have been described previously (11).

RESULTS

Incorporation of ¹⁴C-Palmitate

The rate of phospholipid formation from isotopically labeled precursors has already been reported for whole cells (11). The incorporation of ¹⁴C-palmitate is essentially complete after 5 min and therefore is, in effect, a pulse labeling of lipids synthesized during that time interval. We have now determined the intracellular sites of initial lipid synthesis and the patterns of radioactive lipid movement from one membrane type to another.

Fig. 1 depicts the rates at which 1-¹⁴C-palmitic acid is incorporated into phospholipids of whole cells and fractions thereof. The specific activity data plotted are not corrected for variations in initial cell concentrations, which were unavoidable from one experiment to the next (ideally, the whole cell specific activity would be almost constant for the first hour and decline at 6 and 12 hr as the cell mass increases). The important conclusions to be drawn from Fig. 1 come from comparisons of the specific activities of the different cell fractions at a particular time period.

1 min after the addition of the ¹⁴C-palmitate, the highest specific radioactivity is found in the microsomal supernatant, with the microsomal fraction being slightly but consistently less. Of the other fractions, all of which contain lower radioactivity, the cilia and ciliary supernatant show the most pronounced differences; their specific radioactivities are only 14% that of the microsomal supernatant.

As the time of sampling increases, the microsomal fraction reaches a level of specific activity equivalent to that of the microsomal supernatant. The levels of the remaining membranes rise more gradually. The cilia and the associated fraction are most unusual in requiring a full 6 hr to approach the specific activity of the other cell membranes. During this time 1–2 cell doublings occur.

Although there is significant labeling of triglycerides at the 1- and 5-min intervals, the bulk of radioactivity is found in phospholipids. Analysis of the distribution of radioactivity among the three major phospholipids, namely the choline,

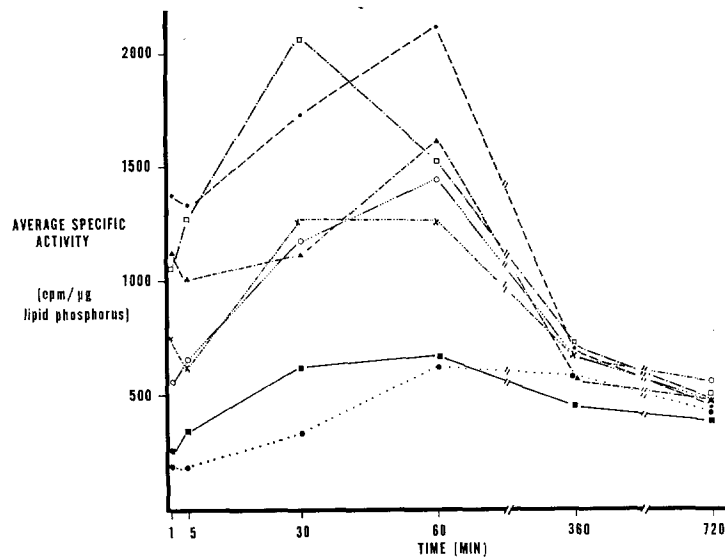


FIGURE 1 The incorporation of $1\text{-}^{14}\text{C}$ -palmitic acid into total phospholipids of individual cell fractions. The tracer was added at 0 min, and incorporation into lipids was essentially complete by 5 min. Phospholipid radioactivity was determined as that remaining at the origin of thin-layer chromatography plates run in the system petroleum ether:ethyl ether:acetic acid (70:30:1). Each point represents the average of three experiments. ●, microsomal supernatant; □, microsomes; ▲, whole cells; ×, pellicles, ○, mitochondria; ■, ciliary supernatant; ●, cilia.

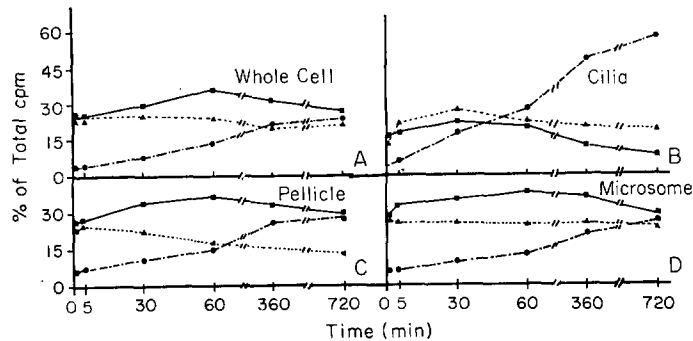


FIGURE 2 Distribution (in percentage of total radioactivity) of ^{14}C among the three principal phospholipids of selected fractions after various time intervals. Each point represents the average of three experiments. ■, ethanolamine phosphatides; ▲, choline phosphatides; ●, 2-aminoethylphosphonolipids.

ethanolamine, and 2-aminoethylphosphonate-containing lipids, reveals an interesting transition. As reported earlier for whole cells (12), each subcellular fraction sustains a much more rapid labeling of the choline and ethanolamine lipids than is observed for the phosphonolipid. Whereas the patterns all indicate the same trend, delayed labeling of the phosphonolipid is most obvious in the ciliary membrane (Fig. 2) and the similar ciliary supernatant, where this lipid is present

in highest concentration (9). Lysophosphatides (including lysophosphonolipid) contaminating the choline phospholipid area on thin-layer plates of the cilia fraction accumulated enough radioactivity by 6 hr to introduce a significant error. Thus the last two points on the curve for choline phospholipids should properly be somewhat lower. Insufficient radioactivity remained to determine accurately the extent of this contamination. The pattern for the whole cell, pellicle,

TABLE I
*The Relative Labeling of Ciliary Lipids and
 Microsomal Lipids after Short and Long
 Incubations with ¹⁴C-Palmitate*

Lipid fraction	Ratio of	
	Specific radioactivity in microsomes	
	Specific radioactivity in cilia	
	1 min	360 min
AEPL	7.9	1.0
PC	15	1.4
PE	4.5	1.2

AEPL, 2-aminoethylphosphonolipids; PC, choline phosphatides; PE, ethanolamine phosphatides.

and microsomal fractions are given for comparison. The mitochondrial and postmicrosomal supernatant fractions resemble, in general, the microsomal pattern. Only after 12 hr do all the three major phospholipids reach equal specific radioactivities.

Although the sluggish ¹⁴C-uptake by phospholipids explains in part the low initial specific radioactivity of the two ciliary fractions, it does not fully account for the depressed labeling rate of the fractions. As shown in Table I, 1 min after labeling, all three major phospholipids are much less radioactive in the cilia than in the microsomal supernatant. This is no longer the case after 6 hr.

When ciliary supernatant from cells labeled 12 hr was further centrifuged at 100,000 *g* for 60 min, the resulting pellet contained two thirds of the recovered radioactivity. The distribution of radioactivity was similar to that of the parent fraction. An additional centrifugation at 200,000 *g* for 60 min produced a small pellet and a supernatant still containing phospholipid. In both of these latter fractions, the radioactivity was approximately equal in the three major phospholipids. However, the presence of considerable non-polar lipid, presumably fatty acids, raises the possibility of lipid degradation. Further characterization of these fractions must be made.

Only limited analysis has been carried out on the postmicrosomal supernatant. Half of the phospholipid of that fraction is sedimented upon recentrifugation at 200,000 *g* for 60 min. The specific activities of the two subfractions have been compared at only one time period, 12 hr after labeling. The specific activities were nearly identi-

cal, as was the distribution of radioactivity among the individual phospholipids.

Incorporation of ¹⁴C-Acetate

The rapidity with which ¹⁴C-palmitate is incorporated suggests that it is being absorbed through the surface membrane. The fatty acids typically used for membrane synthesis are not obtained in this fashion but are synthesized inside the cell from water-soluble precursors. In order to confirm that the ¹⁴C-palmitate pattern is a true indicator of the normal metabolic sequence, we have compared our data with the labeling patterns seen after incubations in the presence of 1-¹⁴C-acetate. Previous experiments have shown that this substrate is incorporated into the fatty acid, glyceryl ether, and tetrahymanol moieties of lipids (11). The pattern of specific activities in lipids of the various cell fractions is given in Fig. 3. The 30-min and 360-min experiments cannot be thought of as strict pulse labeling experiments. Even with the precautions taken to remove excess ¹⁴C-acetate after the 5-min labeling period, incorporation continued at a low rate, probably drawing upon a pool of intracellular acetate.

The specific activities of less active fractions divided by the specific activity of the microsomal supernatant give ratios which are similar to those calculated from the ¹⁴C-palmitate data. Thus the dissimilarities in appearance of the two families of curves are caused by differences in the absolute amounts of label taken up rather than differences in intracellular localization. It is clear that the distribution of ¹⁴C-acetate-labeled lipids follows the same pattern observed in the ¹⁴C-palmitate experiments.

Radioactivity from ¹⁴C-acetate was rapidly incorporated into tetrahymanol. Whereas this compound never accumulated more than 10% of the total radioactivity in most fractions, in cilia it rose from 6% at 5 min to 26% at 360 min.

Incorporation of ³H-Chimyl Alcohol

It is always difficult in studies of this type to deal with lipid exchange processes. Reactions of two sorts can occur: exchange of entire lipid molecules, and exchange of some component of the molecule, such as a fatty acyl group. We have attempted to determine how the latter type of exchange might be influencing our findings.

Three experiments were performed in which

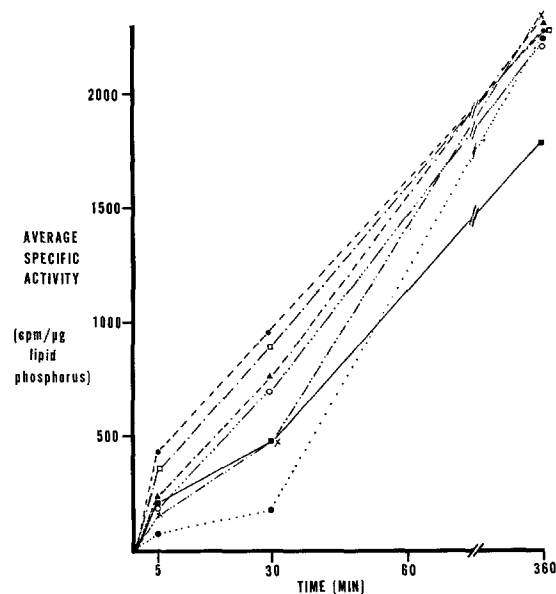


FIGURE 3 The incorporation of $1\text{-}^{14}\text{C}$ -acetate into total phospholipids of individual cell fractions. Phospholipid radioactivity was measured as described in Fig. 1. Each point represents the average of two experiments. The values have been normalized to represent equal cell numbers. Symbols are the same as those used in Fig. 1.

8,9- ^3H -chimylyl alcohol was administered to cells. The substrate was incorporated almost exclusively into phospholipids, and the rate of uptake was so rapid that less than 5% of the ^3H -chimylyl alcohol remained in a free state after 5 min. Under these conditions, there is no significant cleavage of the ether bond (11). Since the labeled side-chain remains bound by its ether linkage to the glycerol backbone of the phospholipid, exchange of the side chain alone is impossible.

Over the first 30 min, the subcellular distribution of radioactivity in these experiments (Fig. 4) is virtually identical to the patterns found with the other two substrates. Thus we may rule out the exchange of acyl side chains as being responsible for the earlier findings. However, it is likely that the exchange of molecular components does have some modulating effect upon membrane lipid distribution. In these latter experiments, where such exchange is prevented, the failure of all fractions to approach a common degree of labeling by 360 min is provocative. In an experiment not included in Fig. 4, the cilia lipids showed a further slight increase in specific radioactivity after 840 min, while the other fractions continued their slow decline.

Over the period during which ^3H -chimylyl alcohol incorporation was followed, the lipids of each cell fraction exhibited the same curious fluctuation of labeling reported earlier for whole cells (12). After 5 min the isolated fractions contained approximately the same percentage of their radioactivity in the choline phosphatides and the phosphonolipids. By 360 min all cell fractions had lost radioactivity from the choline phosphatides while gaining equivalent amounts in the phosphonolipids. Again, the ciliary membranes showed the most striking change, having in the ethanolamine, choline, and aminoethylphosphonate lipids 3%, 5%, and 82% of the label, respectively.

DISCUSSION

Information regarding the mechanisms of membrane synthesis can be obtained by measuring the uptake of radioactive membrane precursors. It has frequently been observed that such precursors are incorporated into functionally distinct membranes of the cell at differing rates. Thus brain myelin shows an apparently slower turnover of lipids than microsomes and mitochondria (13), and labeled endoplasmic reticulum of isolated liver cells seems to donate its radioactive lipids to

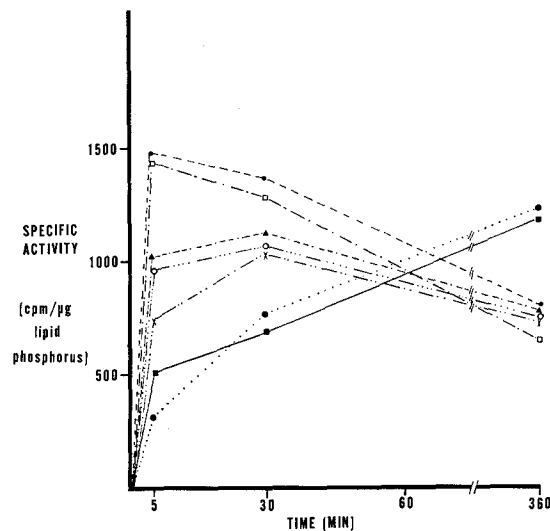


FIGURE 4 The incorporation of 8,9- ^3H -chimyl alcohol into total phospholipids of individual cell fractions. Radioactive medium was replaced by fresh unlabeled medium after 5 min. Phospholipid radioactivity was measured as described in Fig. 1. Each point represents a single experiment. The values have been normalized to represent equal cell numbers. Symbols are the same as those in Fig. 1.

mitochondria (14). Significant differences in incorporation rates can be noted even between the inner and outer membranes of mitochondria (2).

The rate at which added radioactive precursors are incorporated into a given membrane is not necessarily a measure of its rate of synthesis. As pointed out by Warren (15), apparent biosynthetic rates can be erroneous due to the presence of membrane precursor pools, recycling of rejected membrane components, exchange of components between membranes, and other complicating factors.

Because the interactions just described are so poorly understood, it can be misleading to study the biogenesis of one or two membrane types without regard to others present in the same cell. Regrettably, however, few studies involving a wide variety of cellular membranes have been reported. Tsao and Cornatzer (16) compared the incorporation of $^{32}\text{P}_i$ into selected subcellular fractions of HeLa and KB cells, finding all fractions to be approximately equal in specific radioactivity throughout the first 3 hr. A different pattern was briefly reported for *Acanthamoeba* by Chlapowski (17). ^3H -glycerol was incorporated fastest into the postmicrosomal supernatant, and the rough endoplasmic reticulum and nuclear membrane maintained a specific radioactivity generally higher than that of other cell fractions.

In this report we have analyzed the incorporation of lipid precursors into a number of *Tetrahymena* cell fractions which together account for all the membranes of the organism. Our data agree with Chlapowski's findings that significant differences exist in the rates at which particular membranes acquire newly synthesized lipids. The changes noted with the passage of time reveal very clearly that lipid precursors first incorporated into one *Tetrahymena* membrane type later find their way into structurally and functionally different types. The similarity of the labeling patterns after ^{14}C -palmitate and ^{14}C -acetate administration gives us added confidence that we are observing the normal pathways of lipid synthesis. The fact that two compounds so different in physical properties should penetrate to an interior cellular compartment for insertion into phospholipids argues strongly for a specific cellular site of lipid biogenesis. The ^3H -chimyl alcohol incorporation data provide additional support for this concept. In addition, since the labeled side-chain of chimyl alcohol is not cleaved from the glyceryl backbone to a significant degree (11), these latter results imply the intracellular movement of intact phospholipids rather than exchangeable acyl side chains.

Previous studies (18) on the ultrastructure of *Tetrahymena* established that some of the mem-

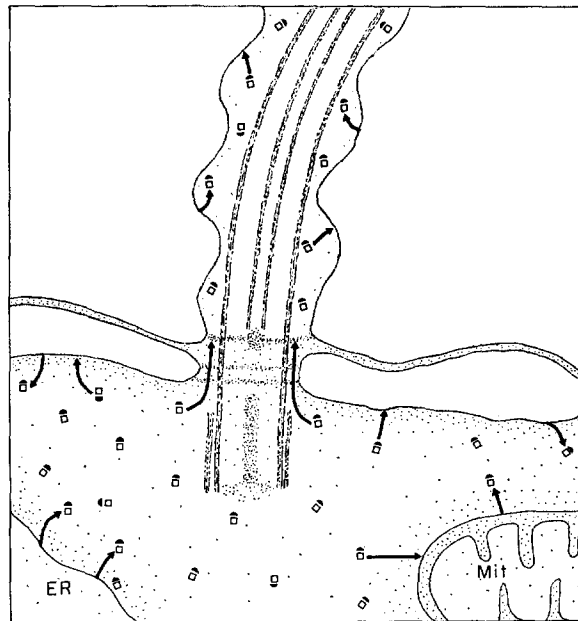


FIGURE 5 The hypothetical movement of membrane lipids within the *Tetrahymena* cell. ER, endoplasmic reticulum; Mit, mitochondrion; \square , lipid-carrier protein complex.

brane system we have analyzed, namely the ciliary and outer pellicular membranes, are physically separated from the main bulk of the cell's cytoplasm by other membranes. Direct access to cytoplasmic constituents can only be had by movement of those constituents through the periodic openings which allow passage of the ciliary shafts into the cytoplasm. We feel that the observed delay in incorporation of newly formed lipids into these outer membranes is due to the membranes' relative inaccessibility. In order to account for their low specific activities measured shortly after the addition of label, we must postulate the existence of a metabolic pool of structural lipids physically isolated from the main cytoplasmic pool. The presence of the pool would explain how ciliary membranes can be fabricated at the same rate (relative to their cellular content) as all other membranes of the growing cells, and yet manifest a lower incorporation of newly synthesized lipid. We tentatively propose the scheme illustrated in Fig. 5.

According to this hypothesis, lipids synthesized for use in membrane expansion are formed predominantly or perhaps even solely by enzymes located in the cytoplasm or endoplasmic reticulum. These newly made lipids are rapidly transported

to all readily accessible cellular membranes, where they are donated or exchanged for preexisting lipids. We can most easily envision the transport of these structural lipids as occurring on a type of carrier protein, such as that reported in rat liver by other authors (5, 6, 7, 8).

The sizeable amount of phospholipid which remains in *Tetrahymena* postmicrosomal supernatant after centrifugation at 200,000 g may be phospholipid complexed with such a carrier protein. Further study will be needed to determine if this fraction participates in intracellular lipid exchange. The addition of lipids to ciliary membranes may occur in the same manner but from the cilium's own carrier proteins whose freedom to equilibrate with the principal cytoplasmic pool is limited by the narrow passageway at the base of each cilium (18). The relatively slow exchange between the two pools of carrier protein could result in the initially low radioactivity in the cilia and ciliary supernatant. We cannot at this time explain the marked enrichment of phospholipids in these fractions.

The significance of the rise in 3H -chimy alcohol-labeled cilia and ciliary supernatant lipids past the specific activity of lipids from the internal membranes is not clear. Certainly, there is a net

movement of phospholipid into the cilia. If the reversible exchange of intact lipids back into the cytoplasm is comparatively slow, an eventual tritium enrichment in cilia would be expected. A more rapid exchange of acyl groups could prevent such an enrichment in the ^{14}C -palmitate and ^{14}C -acetate experiments. On the other hand, the apparent enrichment may not be real, but merely the reflection of a naturally higher glyceryl ether concentration in the ciliary membranes. We have presented preliminary indications (7) that this is not the case, but further studies are very much needed to clarify this point.

If our general hypothesis is correct, the outer pellicular membrane should also be metabolically isolated from the cytoplasm. The middle membrane of the pellicle may be equally isolated, although it has an alternative possibility for growth, namely by an expansive outflow of the inner membrane with which it is continuous. The observed specific activities for the pellicle are intermediate between those of the ciliary membranes and the microsomal membranes. We are hopeful that it will be possible to subfractionate the pellicle and so determine if the three membranes differ in specific radioactivity. At the present time, experiments are in progress to seek the answer to this question by use of electron microscope radioautography. Excellent preservation of the structural features of isolated pellicles (see Fig. 4 of the companion paper) and separations of as much as $1\ \mu$ between the inner pellicular membrane² and the outer two lead us to believe that radioautography will provide useful information.

An important yet poorly substantiated detail of our hypothesis is the role of a carrier protein. We plan to attempt depletion of the cytoplasmic pool of protein-bound lipids by abruptly adding artificial "food," thus inducing the rapid, large-scale formation of food vacuoles (19). Experiments of this type might reveal that the postulated carrier protein itself may be a membrane precursor. The existence of such a precursor pool is suggested by our observations that vacuole formation can continue for some time after protein synthesis is blocked by puromycin.³

The interpretation of isotope labeling experi-

² Labeled objects can be resolved by electron microscope radioautography if they are as as much as $0.1\ \mu$ apart.

³ Thompson, G. A., Jr. Unpublished data.

ments of the type described here is made difficult by our lack of knowledge as to whether lipids exchange out of membranes into which they were initially inserted (5, 6, 7, 8). It is of great importance to determine the magnitude of this exchange, if it exists. Exchange can be measured conveniently by transferring *Tetrahymena* into an inorganic medium capable of sustaining life but not growth (20). The difference in incorporation rates under these conditions and those allowing cell growth should represent the contribution resulting from net synthesis.

Tetrahymena pyriformis is beyond question a versatile tool for the analysis of membrane biogenesis. The observations reported here have already provided some insight into the process of membrane fabrication. The organism's greatest potential lies in studying the control mechanisms invoked during the normal cell cycle. Experiments with synchronously dividing cells should help to explain how growth of various membrane systems is modulated in cell division.

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