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STUDIES OF MEMBRANE FORMATION IN *TETRAHYMENA PYRIFORMIS*

V. LIPID INCORPORATION INTO VARIOUS CELLULAR MEMBRANES OF STATIONARY PHASE CELLS, STARVING CELLS, AND CELLS TREATED WITH METABOLIC INHIBITORS

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SUMMARY

The distribution rates of radioactive phospholipids to various membranes of *Tetrahymena pyriformis* are nearly identical in cells experiencing rapid growth, no growth, and starvation-induced shrinkage. Furthermore, neither the inhibition of protein synthesis by cycloheximide nor the inhibition of fatty acid synthesis by tetralylphenoxyisobutyric acid altered lipid distribution rates. As recently observed in other organisms, lipid insertion into *Tetrahymena* membranes is not coupled to protein insertion, and the reverse situation also seems to be true. The data are best explained by postulating a rapid exchange of intact lipids as being the prime mechanism causing intracellular distribution of these molecules.

INTRODUCTION

The manner in which cells assemble their membranes from protein and lipid constituents is incompletely understood. We have recently described certain features of the ciliate *Tetrahymena pyriformis* which qualify it as a most promising model system for studying membrane biosynthesis, particularly with regard to the metabolic interrelationships existing between functionally different membranes coexisting within the same cell (refs 1–3 and L. C. Baugh and G. A. Thompson Jr., unpublished results).

Our experiments with cultures of *Tetrahymena* in logarithmic growth showed an initial rapid incorporation of radioactive lipid precursors into certain cytoplasmic membranes, while the surface membranes required a much longer time to attain a comparable level of radioactivity. Although the data clearly showed a movement of newly synthesized lipids from the inner compartments of the cell to the outer regions, the mechanism by which the molecules were transported could not be determined.

Using these rapidly growing cells, there was difficulty in deciding the extent to

Abbrevation: TPIA, tetralylphenoxyisobutyric acid.

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which the observed movement of newly synthesized lipids into the various membrane fractions is caused by a net lipid addition to these membranes, as opposed to a replacement of new lipid molecules for old ones, perhaps involving a one-for-one exchange reaction of the type described in rat liver preparations by Wirtz and Zilversmit[4] and in intact rat liver cells by Jungalwala and Dawson[5]. The answer to this question has been sought by studying cells under culture conditions in which there is no net membrane synthesis. This communication describes results indicating that rapid inter-membrane lipid exchange is an important factor in membrane metabolism.

METHODS

T. pyriformis, strain E, was cultured at 24 °C in an enriched proteose peptone medium as previously described[6]. In experiments involving logarithmic phase cells, 200-ml cultures were utilized after 39–42 h of growth on a rotary shaker, at which time the cells had reached middle logarithmic phase (approximately $4 \cdot 10^5$–$6 \cdot 10^5$ cells per ml). The cultures were quickly chilled and harvested by centrifugation as described earlier[1]. Subcellular organelles were isolated according to our previously published procedure[1]. Most organelle fractions yielded 30–50 μg lipid phosphorus, but analysis of the least plentiful fraction (ciliary supernatant) was generally made using 4–8 μg lipid phosphorus.

In some experiments, the logarithmic phase cells were resuspended in 200 ml inorganic medium[7] after their initial centrifugation. They were recentrifuged at 164 $\times$ *g* for 5 min. The pellet was washed once more by resuspension in inorganic medium and centrifugation, and was then resuspended in 500 ml fresh inorganic medium for continued incubation with shaking at 24 °C. Because aliquots of these cultures maintained in the absence of a carbon source contained unusually small cells, they were harvested by centrifugation for 5 min at a higher force, 2000 $\times$ *g*.

For those experiments requiring stationary phase cells, 200-ml cultures were grown to an age of approximately 60 h, at which time the cell density averaged $1.5 \cdot 10^6$ cells per ml.

Cell counts were made using a hemocytometer. Lipids were extracted by the method of Bligh and Dyer[8] with the final chloroform phase being washed two times by the method of Folch *et al.*[9] to remove non-lipid impurities. Thin-layer chromatography was carried out as described previously[1]. Lipid phosphorus was determined by the procedure of Bartlett[10] as modified by digestion with perchloric acid according to Marinetti[11]. Protein was measured by the method of Lowry *et al.*[12], using bovine serum albumin as a standard.

Sodium [1-^{14}C]acetate (55 mCi/mmole) and [1-^{14}C]palmitic acid (54 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass, and [9,10-$^3H_2$]-palmitic acid (500 mCi/mmole) from Amersham-Searle Corp., Arlington Heights, Ill. The labeled palmitic acid ($1 \cdot 10^6$–$2 \cdot 10^6$ cpm) was administered by dissolving the compound in 2–4 drops absolute ethanol and injecting it below the surface of a rapidly stirred culture with a Pasteur pipette[1]. The [^{14}C]acetate, carrier-free $H_3{}^{32}PO_4$ (Schwarz-Mann, Orangeburg, N.Y.) and L–[4,5-$^3H_2$]leucine (29.8 Ci/mmole, International Chemical and Nuclear Corp., Irvine, Calif.) were administered in aqueous solution. All samples were assayed for radioactivity using a Packard Instrument Co.

Model 3310 scintillation spectrometer having a counting efficiency of 89% for ^{14}C and 49% for ^{3}H. Proteins were prepared for isotope assay by a modification of the procedure of Byfield and Scherbaum[13].

Tetralylphenoxyisobutyric acid (TPIA)[14] was a generous gift from Dr M. E. Maragoudakis. Cycloheximide was a Sigma product.

Isopycnic density gradient centrifugation was performed on microsomal preparations from cycloheximide-treated cells given [^{14}C]palmitate 10 min prior to harvest and 0.1 mM TPIA-treated cells labeled with [^{3}H]palmitate 10 min before harvest. The microsomal membranes were isolated separately from the two cultures, then mixed and sonicated for 5 min in 2 ml of chilled standard phosphate buffer[1] at a setting of 4 on a Branson Model W140 Sonifier. Aliquots of the mixed microsomal fragments were layered on a linear sucrose-density gradient (10–30% sucrose in buffer). After isopycnic equilibrium was attained (100000 × *g*, 150 min), the tube was fractionated into 160-μl portions, each of which was monitored for protein (absorption at 280 nm) and for radioactivity.

RESULTS

Intracellular lipid transport in non-growing cells

Most experiments described in our previous communications[1-3] have involved cells in the logarithmic phase of growth, under conditions where the mass-doubling time is 3–4 h. It was of interest to compare the rates of intracellular lipid distribution in the growing cells with those in cells existing in a non-growing state. Non-growing cells were obtained in two ways. One was by allowing the cell density of normal cultures to increase until cell division ceased and the culture entered the stationary phase. In this phase the cell density remained constant for approximately 100 h[15]. Alternatively, cells were placed in a medium containing inorganic salts but no carbon source[7]. In the inorganic medium cell division and fatty acid synthesis ceased within 4–5 h but the cells remained motile and viable for several days. During this time there was a gradual reduction of cell volume to approximately 20% of the normal value[15]. This decrease in size was accompanied by an equivalent decrease in lipid per cell.

Using either type of non-dividing culture, it was possible to determine the rate of [^{14}C]palmitate incorporation into lipid and the redistribution of this lipid throughout the cell. Just as in logarithmic phase cells[2], incorporation of the isotope into lipids was very rapid, being nearly complete after 10 min. While most of the radioactivity was found in phospholipids (Fig. 1), significant levels of ^{14}C were also found in triglycerides in the non-growing cells.

Samples of starving cells were harvested at each time interval shown in Fig. 1 and fractionated into cilia, ciliary supernatant, pellicles, mitochondria, microsomes, and post-microsomal supernatant as previously described[1]. Fig. 2 illustrates the labeling pattern observed. As found earlier with rapidly growing cells, there was a marked difference in specific radioactivity of the various fractions after brief exposures to [^{14}C]palmitate. The microsomes and the post-microsomal supernatant contained lipids initially having specific activities much higher than the other isolated fractions. However, with the passage of time, radioactive lipids were deployed from microsomes and post-microsomal supernatant to the other cell structures, so that by 12 h all lipid-containing regions were nearly equally labeled. When compared with the

equivalent data obtained using logarithmic phase cells[2], phospholipids of different fractions from starving cells follow a similar pattern, but the fractions approach an isotope equilibration at a slower rate, particularly in the case of cilia and ciliary supernatant.

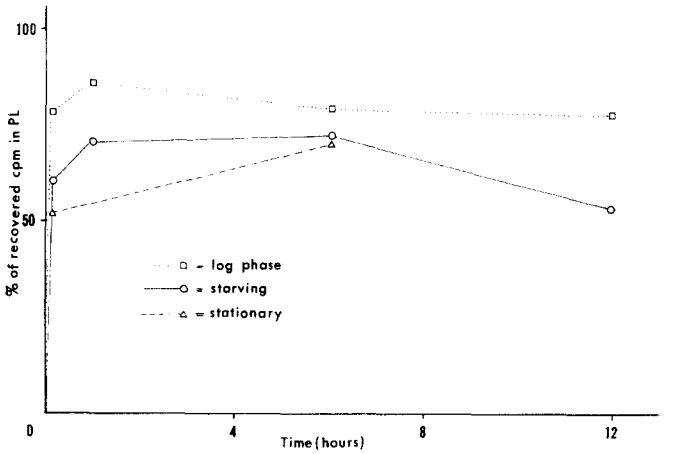

Fig. 1. Rates of [^{14}C]palmitate incorporation into the whole cell phospholipids (PL) of logarithmic phase, starving, and stationary phase *Tetrahymena*. After incorporation was complete (< 1 h), the non-phospholipid radioactivity was present in the form of triglycerides.

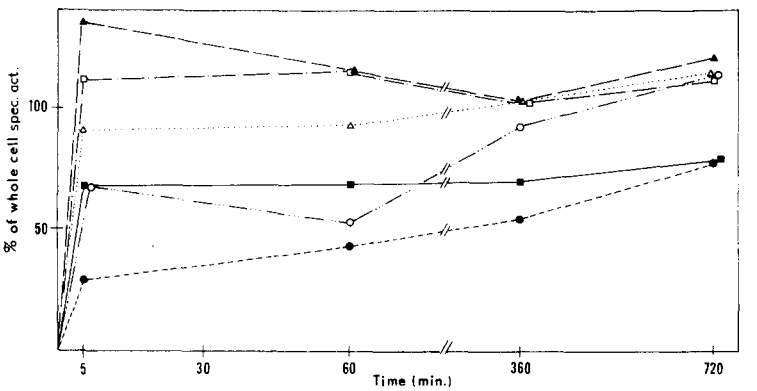

Fig. 2. Relative specific radioactivities of various subcellular fractions isolated from starving cells at different times following the administration of [^{14}C]palmitate. Each point represents one experiment, except points at 5 and 720 min, which are averages of duplicate experiments. The average specific radioactivity of whole cell lipids in all experiments was 1100 cpm/μg lipid phosphorus. ●, cilia; ■, ciliary supernatant; △, pellicles; ○, mitochondria; □, microsomes; ▲, post-microsomal supernatant.

Analysis of the individual lipid fractions after separation by thin-layer chromatography revealed a distribution of radioactivity unlike that found in growing cells. For one thing, a higher percentage (10–30 %) of ^{14}C appeared in triglycerides than occurred in logarithmic phase cells (< 10 %). Among the phospholipids, starving cells contained a lower proportion of radioactivity in phosphonolipid than did logarithmic phase cells. Table I illustrates this trend in whole cell lipids and in cilia,

the organelle most enriched in phosphonolipid. The other cell fractions exhibited the same tendency.

Data of a similar nature were obtained using cells in the stationary phase of growth. Under the culture conditions employed in this study, cells cease dividing after approximately 60–70 h of growth at a cell density approaching $2 \cdot 10^6$ cells per ml. After that time no further increase in phospholipid occurs, although triglycerides* accumulate for some hours. Fatty acid synthesis is drastically curtailed[15]. Membranes of stationary phase cells exist in a steady state characterized by low but balanced rates of lipid and protein synthesis and degradation.

TABLE I

PERCENTAGES OF TOTAL RECOVERED RADIOACTIVITY FOUND IN THE MAJOR PHOSPHOLIPIDS 5 min OR 720 min AFTER ADMINISTRATION OF [^{14}C]PALMITATE

	Whole cells		*Cilia*	
	Fed	*Starved*	*Fed*	*Starved*
Labeling pattern after 5 min				
Phosphatidylcholine	25	18	24	12
Phosphatidylethanolamine	25	21	16	14
2-Aminoethylphosphonolipid	5	2	7	2
Labeling pattern after 720 min				
Phosphatidylcholine	21	8	16	16
Phosphatidylethanolamine	28	27	9	19
2-Aminoethylphosphonolipid	23	8	59	27

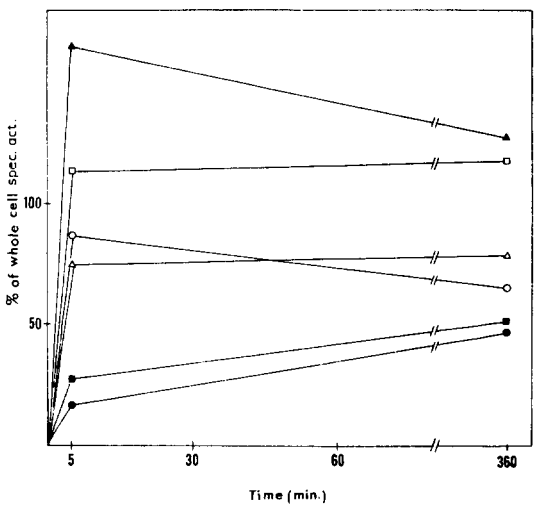

Fig. 3. Relative specific radioactivities of various subcellular fractions isolated from stationary phase cells at the indicated times following the administration of [^{14}C]palmitate. Each point represents data from one experiment. The average specific radioactivity of whole cells was 620 cpm/μg phosphorus. Symbols are as shown in Fig. 2.

* The triglycerides synthesized under these conditions were recovered primarily in the post-microsomal supernatant.

Cultures 95–100 h old were incubated with [^{14}C]palmitate for 5 min or 360 min and then fractionated. The incorporation of radioactivity into phospholipids of the cell components is shown in Fig. 3. The rates of ^{14}C movement from cytoplasmic membranes to surface membranes were slightly less than in inorganic medium-grown cells, but the patterns were generally similar in both cases.

Metabolic stability of lipids under various culture conditions

We presented data earlier showing that in rapidly growing cells the patterns of intracellular lipid movement are the same whether one follows the hydrocarbon side chains or the glycerol backbone (as measured with alkyl glyceryl ethers)[2]. This suggested to us that lipids are transported as intact molecules. In the experiments reported above, however, added [^{14}C]palmitate is incorporated into phospholipids of cells experiencing no net phospholipid increase, indicating at least some degree of lipid degradation and resynthesis.

In trying to visualize the transport mechanisms operative in the cells, it is important to gain some quantitative impression of this metabolic turnover and to learn whether its rate varies with the physiological state of the cell. Gauging turnover by analysis of fatty acid radioactivity is misleading because those molecules released during degradation may be immediately reutilized. We chose instead to examine the stability of ^{32}P-labeled lipids. Logarithmic phase cells were grown for 1 h in the presence of 0.8 μCi carrier-free $^{32}P_i$ per ml, then washed free of $^{32}P_i$ and resuspended in fresh medium containing a 10^8-fold excess of non-radioactive phosphate. Samples were extracted for lipids at various time intervals. The results from three comparable experiments indicated a 30 % initial increase of lipid radioactivity during the first 2 h following the chase, indicative of a utilization of the internal $^{32}P_i$ pool. During the next 4 h there was an average loss of 13 % of the total lipid radioactivity, followed by a much slower loss during the subsequent 24-h period. The specific radioactivity decreased steadily throughout this period due to a rapid net synthesis of lipid containing unlabeled phosphorus.

A $^{32}P_i$-pulse–chase experiment in which the culture was sampled both before and after it entered stationary phase revealed that essentially all radioactive phosphorus was retained for at least 12 h in the non-growing condition. In addition, pulse-labeled cells were resuspended in inorganic medium to induce starvation and were analyzed for metabolic turnover as the ensuing net depletion of lipids began. Although an absolute diminution of radioactivity was detected, this could be attributed to the net reduction of the phospholipid content. The specific radioactivity, which in the starving cells is a better measure of turnover, decreased by 9–22 % after 6 h and 24 % after 8 h. By this criterion, then, both growing and non-growing cells contain phospholipids whose phosphate-containing backbones, at least, are remarkably stable with relation to the rates at which the lipids are being transferred from site to site within the cell.

The effects of metabolic inhibitors on intracellular lipid transport

It seemed desirable to expand our study of the lipids themselves to include a scrutiny of possible mechanisms responsible for their mobility. Since proteins constitute a major portion of membrane structure, it was logical to begin by testing for a relationship coupling movement of these two principal membrane components.

Accordingly, we tested the mobility of membrane lipids under conditions where protein synthesis was selectively inhibited. Cycloheximide has been shown to block protein synthesis in *Tetrahymena* very effectively[16]. Under our conditions, a concentration of 100 μg cycloheximide per ml immediately stopped the incorporation of [^{3}H]leucine by logarithmic phase cells. Although this inhibition continued to be exerted for at least 4 h, fatty acid synthesis from [^{14}C]acetate continued at a normal rate. During this initial period of inhibition, added [^{14}C]palmitate was incorporated into glycerides in the normal way, but after 4 h a lowered rate of palmitate utilization indicated either a decrease in the demand for new lipid or a reduction in the enzymatic capacity for lipid synthesis.

Table II presents selected findings from several experiments involving cycloheximide inhibition. The data for microsomes and cilia as well as those for the other subcellular fractions (not shown) lead to the conclusion that the transport of radioactive lipids throughout the cell is not significantly affected by inhibition of protein biosynthesis.

TABLE II

EFFECTS OF CYCLOHEXIMIDE ON INTRACELLULAR LIPID TRANSPORT

Logarithmic phase cells were fractionated after treatment with 100 μg cycloheximide per ml and $1 \cdot 10^6$ cpm of [^{14}C]palmitate at the times indicated. Whole cell lipids averaged 1290 cpm/μg lipid phosphorus.

Experimental conditions	*Spec. act. (% of whole cells)*	
	Microsomes	*Cilia*
25 min cycloheximide pretreatment + 5 min with ^{14}C	142	14
360 min cycloheximide pretreatment + 5 min with ^{14}C	108	40
5 min ^{14}C, then 55 min cycloheximide	119	48
5 min ^{14}C, then 360 min cycloheximide	91	92
5 min ^{14}C, then 360 min cycloheximide	100	93
Control, 5 min with ^{14}C	130	18
Control, 360 min with ^{14}C	136	100

It was also possible to measure the intracellular redistribution of lipids within logarithmic phase cells in which lipid synthesis was inhibited. Such an analysis could be made by employing the recently described inhibitor potassium tetralylphenoxyisobutyric acid (TPIA)[14]. This compound appears to be a specific inhibitor of acetyl-CoA carboxylase in higher animals[17]. In logarithmic phase *Tetrahymena* cells, the effect of TPIA was instantaneous and pronounced. When tested at a concentration of 0.1 mM, the compound produced a 90 % inhibition of [1-^{14}C]acetate incorporation into *Tetrahymena* lipids (Table III).

When tested at a higher concentration (0.2 mM), the incorporation of [^{14}C]-acetate into lipids was immediately reduced to 2 % of controls, and the cells' shape soon became very rounded. TPIA at a lower concentration (0.05 mM) produced an 80 % inhibition [^{14}C]acetate conversion to lipids, but recovery began within one hour, and after 6–8 h normal cell division resumed.

The decrease in synthesis induced by 0.1 mM TPIA was found fairly uniformly in all lipids, including tetrahymanol. After a 4-h exposure to the inhibitor, the small

amount of radioactivity that could be incorporated into lipids during a 30-min incubation was distributed in an abnormal manner, with almost equal amounts entering phospholipids and triglycerides (normal cells incorporate 7–8 times more radioactivity into phospholipids than into triglycerides). The inhibited cells remained motile but became somewhat rounded in shape and were not observed to divide for at least 7 h.

TABLE III

THE EFFECT OF 0.1 mM TPIA ON THE INCORPORATION OF [1-^{14}C]ACETATE INTO LIPIDS BY $14 \cdot 10^6$ LOGARITHMIC PHASE CELLS

TPIA was added at 0 min and 5 μCi tracer was added at the times indicated. All samples, including the control, were incubated with the tracer for 30 min.

Experimental conditions	*Incorporation into lipids (cpm × 10^{-5})*
[^{14}C]Acetate added at 0 min	1.05
[^{14}C]Acetate added at 60 min	1.03
[^{14}C]Acetate added at 240 min	1.73
Uninhibited control	15.22

Despite these effects, added [^{14}C]palmitate could still be rapidly incorporated into lipids 5 or 25 min following TPIA addition (although by the latter time over half the incorporation was already into neutral lipids rather than phospholipids).

Several experiments were conducted to test the effect of this inhibitor on intracellular lipid mobility. Logarithmic phase cells were preincubated with [^{14}C]-palmitate for either 10 or 30 min and then were treated with 0.2 mM TPIA for a 3.5-h period before isolating the various membrane fractions. The distribution pattern of radioactivity among the fractions was indistinguishable from that observed in uninhibited cells grown with [^{14}C]palmitate for the same time period. The lower concentration of TPIA (0.1 mM) produced similar results when added after a 30-min pretreatment with [^{14}C]palmitate. One may conclude from these data that fatty acid synthesis is not necessary for intracellular lipid movement.

Thus suspending the net synthesis of membrane structural units, either by stopping cell growth through starvation or overcrowding or by selectively inhibiting a particular metabolic pathway, has no pronounced effect on intracellular lipid movement. The findings prove that migration and insertion of lipid molecules into membranes are not conditioned upon the *de novo* formation of a mobile aggregate of lipid and protein.

Effects of inhibitors upon membrane composition

In the cycloheximide-treated cells described above, lipid synthesis persisted at a nearly normal rate for some time after protein synthesis was stopped. In such cells the lipids should either continue to enter the membranes, eventually creating an abnormally low protein/lipid ratio, or else be shunted into some non-membranous compartment of the cell. The latter alternative is not the case, because experiments involving the isolation of subcellular fractions from long term cycloheximide-inhibited cells (described in Table II) revealed distributions of both lipid phosphorus and lipid

radioactivity similar to those found in uninhibited cells. On the other hand, quantitative analysis of whole cell protein and lipid phosphorus from pairs of matched cultures one of each pair inhibited by cycloheximide, demonstrated a decrease in the protein/phospholipid ratio (Table IV).

TABLE IV

EFFECT OF CYCLOHEXIMIDE (100 μg/ml) UPON THE WHOLE CELL PROTEIN/PHOSPHOLIPID RATIO, AS MEASURED ON A WEIGHT BASIS, ASSUMING PHOSPHORUS TO BE 4% OF THE PHOSPHOLIPID WEIGHT

Cycloheximide added at times indicated and cells harvested after an additional 4 h (5 h in Expt 2).

Expt	*Age of cultures (h)*	*Protein/phospholipid*	
		Control	*Cycloheximide*
1	35	7.1	6.1
2	36	7.1	6.1
3	39.5	5.9	5.8

Similar experiments were carried out using TPIA, the inhibitor of fatty acid biosynthesis. In this case, however, the results were variable, in some cases showing an enrichment in protein and in other cases not. Subsequent investigation showed that the higher concentrations of TPIA tested (0.2 mM) almost completely blocked the incorporation of [^{3}H]leucine into cellular protein. A slight reduction of [^{3}H]-leucine conversion into protein was effected by 0.1 and 0.05 mM TPIA. No data were obtained to indicate what effect TPIA has on [^{3}H]leucine incorporation into specific membrane proteins.

In order to gain a clearer impression of the density changes occurring in mem-

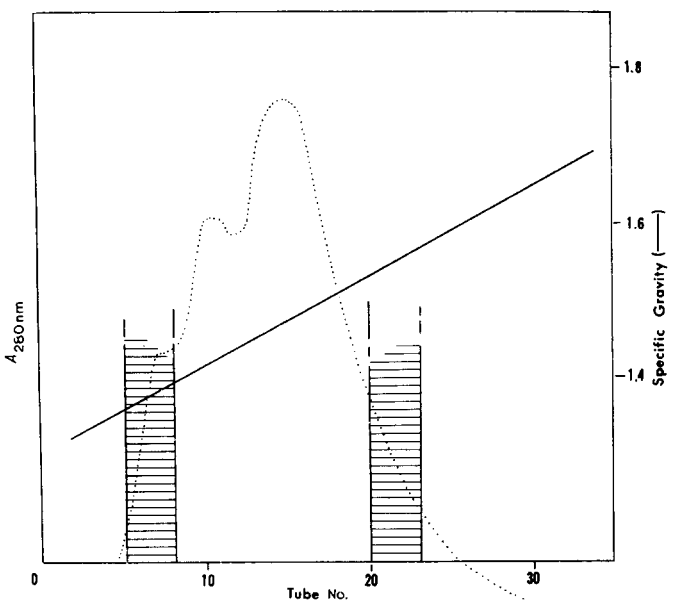

Fig. 4. Absorbance pattern of microsomal fragments banded by isopycnic centrifugation as described in Methods. The hatched bars indicate which parts of the gradient, tubes 5–7 (upper band) and 20–22 (lower band) were pooled for radioactivity assay.

branes proper, we subjected inhibited cells to one further analysis. Logarithmic phase cells exposed for 4.5 h to cyclohexmide were labeled for 10 min with [^{14}C]-palmitate. An identical number of cells was treated for 4.5 h with 0.1 mM TPIA and then labeled for 10 min with [^{3}H]palmitate. The microsomes isolated from each culture were mixed, and the mixture was sonicated. The preparation was then submitted to isopycnic sucrose density gradient centrifugation as outlined in Methods. Analysis of the resulting band of equilibrated microsomal fragments (Fig. 4) revealed that the top zone of the band (lowest density, Tubes 5–7) had a ^{3}H/^{14}C ratio of 1.2 while the bottom zone (highest density, Tubes 20–22) had a ^{3}H/^{14}C ratio of 1.8. Thus, the density of microsomal membrane fragments from the TPIA cells was relatively higher than that of analogous membrane fragments from cycloheximide cells as expected on the basis of the protein/phospholipid ratios.

DISCUSSION

The mechanism employed by cells for inserting newly synthesized structural proteins and lipids into cellular membranes is not known. The lipids in particular, because of their very limited water solubility, would seem to require some means other than simple diffusion to traverse the cytoplasm from their site of synthesis to their membrane destination. It is this movement and its regulation that we seek to elucidate.

Our earlier analyses of membrane expansion in actively growing *Tetrahymena* revealed that newly made lipids are transferred from the endoplasmic reticulum to the outlying regions of the cell at clearly defined rates[2]. Similar radioactivity patterns observed after labeling with [^{14}C]palmitate, [^{14}C]acetate, or [^{3}H]chimyl alcohol[2] or with $^{32}P_i$ (Y. Nozawa and G. A. Thompson Jr, unpublished results) indicated that the migration is primarily one of intact lipid molecules rather than molecular components. New information regarding the metabolic turnover of ^{32}P-labeled phospholipids shows that the phosphate moiety is stable, both in growing and non-growing cells. The ^{32}P-incorporation data strengthen earlier evidence that phospholipid molecules experience relatively little turnover during the periods under study. All the biochemical data thus far accumulated imply that apart from the sharply curtailed production of lipid precursors, metabolism of membrane lipids continues in non-growing cells along the same general pattern operative in rapidly growing organisms. Analyses of the specific activities of individual ^{32}P-labeled phospholipids (to be presented elsewhere) indicate that in all physiological conditions examined thus far there is a comparatively slow interconversion of nitrogenous bases and a somewhat more rapid exchange of fatty acid side chains as the phospholipid molecules traverse the cell from biosynthetic sites to membrane destinations.

In order to further our aim of understanding membrane dynamics in *Tetrahymena*, we have studied lipid mobility under several physiological conditions. The results show that these lipids are distributed to the various membranes at similar rates regardless of whether the cell is experiencing rapid membrane expansion, no change in membrane content, or a net reduction in membrane expanse. Although growing cells do redistribute radioactive lipids at a somewhat faster rate, it seems clear that the observed intracellular dissemination of lipids is not principally due to a net movement of new molecules towards areas of membrane growth. Rather, the

more prevalent mechanism for lipid insertion into a membrane is by the exchange of an incoming lipid for a preexisting lipid, and the differences in rates of labeling reflect the degrees to which the various membranes participate in the exchange process.

Studies with cycloheximide demonstrated that the dissemination of newly synthesized lipid does not depend upon a concurrent synthesis of protein. If proteins are involved, for example as a carrier for lipids, then they must exist in a slowly metabolized pool subject to extensive reutilization. TPIA, the inhibitor of fatty acid synthesis, also produced no significant effect on lipid movement. In this case, the interpretation is less clear, for some phospholipid synthesis from pre-existing fatty acids could have taken place, and the dependence of lipid transport on the formation of new lipids cannot excluded with certainty. However, there is no doubt that net lipid synthesis is almost eliminated by the presence of TPIA, and this process can be ruled out as a driving force promoting intracellular lipid dispersal.

The long-term effect of growth in the presence of cycloheximide is a decrease in the cellular protein/phospholipid ratio. This was demonstrated by direct analysis of whole cells. The opposite effect, an increase in the protein/phospholipid ratio, was noted in some TPIA experiments. The poor reproducibility of these effects renders their interpretation questionable, especially since TPIA has been found to depress protein synthesis as well as lipid synthesis. Density changes in microsomal fragments from inhibited cells subjected to isopycnic density gradient centrifugation confirmed that the density alterations occur in membranes. These results agree closely with the findings that amino acid incorporation into *Staphylococcus aureus* membranes continues in the absence of lipid synthesis[20] and that membrane lipid synthesis is not blocked when protein synthesis is inhibited in *Mycoplasma*[18] and rat brain[19]. Thus, it appears that in *Tetrahymena* too, there is no obligatory coupling between lipid and protein insertion into membranes.

In cells having an altered ratio of protein to lipid because of long-term selective inhibition, movement of freshly synthesized structural lipids into cellular membranes continues at the normal rates. We may conclude that the rates of intracellular lipid movement are not significantly influenced by a requirement for maintaining the protein/lipid ratio within optimal limits.

Our present findings provide no information regarding the precise mechanism by which lipids are transported throughout the cell. However, they do permit an evaluation of several potential factors for regulating lipid transport. Our evidence implies that the replacement of old lipids by new in *Tetrahymena* membranes is a passive process limited only by physical barriers which restrict the access of the new lipids to some compartments of the cell. The relative rate of exchange in these less accessible regions may be no lower than in other compartments, but such an exchange would have to be with "free lipids" of an isolated pool existing in that part and mixing only slowly with the major "free lipid" pool of the central cytoplasm.

The similar labeling patterns found in *Tetrahymena* cells whether they be growing, maintaining a constant size, or shrinking, suggest to us that in this cell the movement of radioactive lipids to various membranes occurs via a relatively small transport vehicle, such as a protein or lipoprotein, and not by the bulk transport of much larger membranous elements[21]. We are currently trying, with some initial success, to characterize a protein carrier of membrane lipids. Concurrently, we are investig-

ating, under the diverse physiological states described above, the dynamics of proteins which serve a structural role in the various intracellular membranes.

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REFERENCES

1 Y. Nozawa and G. A. Thompson Jr, *J. Cell Biol.*, 49 (1971) 712.
2. Y. Nozawa and G. A. Thompson Jr, *J. Cell Biol.*, 49 (1971) 722.
3 G. A. Thompson Jr, R. J. Bambery and Y. Nozawa, *Biochemistry*, 10 (1971) 4441.
4 K. W. A. Wirtz and D. B. Zilversmit, *J. Biol. Chem.*, 243 (1968) 3596.
5 F. B. Jungalwala and R. M. C. Dawson, *Biochem. J.*, 117 (1970) 481.
6 G. A. Thompson Jr, *Biochemistry*, 6 (1967) 2015.
7 K. Hamburger and E. Zeuthen, *Exp. Cell Res.*, 13 (1957) 443.
8 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
9 J. Folch, M. Lees, and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
10 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
11 G. V. Marinetti, *J. Lipid Res.*, 3 (1962) 1.
12 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
13 J. E. Byfield and O. H. Scherbaum, *Anal. Biochem.*, 17 (1966) 434.
14 M. E. Maragoudakis, *J. Biol. Chem.*, 244 (1969) 5005.
15 G. A. Thompson Jr, R. J. Bambery and Y. Nozawa, *Biochim. Biophys. Acta*, 260 (1972) 630.
16 J. Frankel, *J. Cell Physiol.*, 74 (1969) 135.
17 M. E. Maragoudakis, *J. Biol. Chem.*, 246 (1971) 4046.
18 I. Kahane and S. Razin, *Biochim. Biophys. Acta*, 183 (1969) 79.
19 J. A. Benjamins, N. Herschkowitz, J. Robinson and G. M. McKhann, *J. Neurochem.*, 18 (1971) 729.
20 L. Mindich, *J. Mol. Biol.*, 49 (1970) 433.
21 W. W. Franke, D. J. Morré, B. Duemling, R. D. Cheetham, J. Kartenbeck, E.-O. Jarasch and H.-W. Zentgraf, Z. Naturforsch., 26b (1971) 1031.