ASSEMBLY OF LIPIDS INTO MEMBRANES IN
ACANTHAMOEBA PALESTINENSIS

II. The Origin and Fate of Glycerol-3H-Labeled
Phospholipids of Cellular Membranes

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ABSTRACT

The membranes of Acanthamoeba palestinensis were studied by examination in fixed cells, and then by following the movements of glycerol-3H-labeled phospholipids by cell fractionation. Two previously undescribed structures were observed: collapsed cytoplasmic vesicles of cup shape, and plaques in food vacuole and plasma membrane similar in size to the collapsed vesicles. It appeared that the plaques formed by insertion of collapsed vesicles into membranes and/or that collapsed vesicles formed by pinching off of plaques. Fractions were isolated, enriched with nuclei, rough endoplasmic reticulum (RER), plasma membrane, Golgi-like membranes, and collapsed vesicles. The changes in specific activity of glycerol-3H-labeled phospholipids in these membranes during incorporation, turnover, and after pulse-labeling indicated an ordered sequence of appearances of newly synthesized phospholipids, first in nuclei and RER, then successively in Golgi membranes, collapsed vesicles, and finally, plasma membrane. In previous work we had found no large nonmembranous phospholipid pool in A. palestinensis. These observations are consistent with the hypothesis that membrane phospholipids are synthesized, perhaps as integral parts of membranes, in RER and nuclei. Subsequently, some of the newly synthesized phospholipids are transported to the Golgi complex to become integrated into the membranes of collapsed vesicles, which are precursors of the plasma membrane. Collapsed vesicles from the plasma membrane by inserting into it as plaques. When portions of the plasmalemma from food vacuoles, collapsed vesicles pinch off from their membranes and are recycled back to the cell surface.

INTRODUCTION

The origin of cellular membranes is unknown. Specifically, although much is known concerning their chemical components, it is not known where their building blocks are assembled (1). Are constituent molecules added onto preexisting membranes wherever additional membrane is needed (2, 3, 4, 5, 6, 7) or is there a central site where basic membrane structures are synthesized, to be transported, with selective additions and deletions of molecules, to places where other cellular membranes are needed (8)? There is evidence that some of the phospholipid
and protein constituents of membranes are synthesized in the endoplasmic reticulum (8, 9, 10), and it has been observed that membranes are plastic—they can break apart from and fuse with other membranes, as in pinocytosis (11, 12, 13, 14, 15, 16), phagocytosis (17, 18, 19), or the expulsion of a secretory product at the cell surface (20, 21). Transitions in thickness of membranes across stacked Golgi cisternae, from endoplasmic reticulum-like to plasma membrane-like, have prompted the suggestion that the Golgi apparatus is a site for transformation of membrane (22, 23, 24, 25).

Phospholipids are an integral component of all cellular membranes (1); therefore, studies utilizing radioactively labeled phospholipids would be expected to shed light on the biogenesis of membranes. Although there have been studies of the incorporation of phospholipids into membranes, as followed by cell fractionation, the investigators examined only the rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) (3) or microsomes and mitochondria (10).

This report outlines a cell fractionation technique which allows the separation of nuclei, RER, plasma membrane, and Golgi-like membrane fractions. In addition, the isolation of previously undescribed collapsed vesicles from the cytoplasm of A. palestinensis is outlined.

The preceding paper (26) presented evidence that glycerol-3H is a specific marker for membrane phospholipids in these cells. The present paper describes the labeling of newly formed membrane phospholipids and their fate as followed by cell fractionation.

**METHODS**

**Cell Fractionation**

Suspensions of A. palestinensis were cultured axenically in a proteose peptone-glucose medium as described previously (26). The cells used in all experiments grew exponentially with a doubling time of 27 hr, as determined by direct counting.

**All steps of the cell fractionation were carried out at 0°-4°C, at pH 7.5. Medium with growing cells was chilled at the appropriate experimental time by the addition of 2 volumes of an ice-cold solution of 0.25 M sucrose-TM (0.005 M Tris and 0.002 M MgSO4). 2-4 ml of packed cells were harvested and washed twice in 0.25 M sucrose-TM solution by centrifugation at 125 g for 2 min. The washed cells were resuspended to 15 ml with 0.25 M sucrose-TM and homogenized with seven strokes in a 30 ml Potter-Elvehjem glass-Teflon grinder at 2500 rpm. This was sufficient to break about 90% of the cells while leaving most (~90%) of the nuclei intact. The following cell fractions were isolated in sequence in a Sorvall RC-2 refrigerated centrifuge and a Spinco L2-50 ultracentrifuge.

**Nuclear and Plasma Membrane Fractions:** After centrifugation of the homogenate at 2000 g for 15 min in a Sorvall HB-4 swinging-bucket rotor a two-layered pellet was observed. A dark-brown pellet containing mainly unbroken cells was overlaid by a loosely packed white layer containing nuclei, cell-surface "ghosts", and ghost fragments, contaminated with microsomes and mitochondria. The "2000 g supernatant" was decanted for further subfractionation, and the sides of the centrifuge tube were wiped clean with tissues. The white layer was gently and carefully resuspended in 0.25 M sucrose-TKM (0.005 M Tris, 0.025 M KCl, and 0.002 M MgSO4) so as not to disturb the underlying dark pellet. KCl and Mg++ were included to prevent nuclear swelling (27) and clumping (28), respectively. The suspension was washed by centrifugation at 2000 g for 15 min in the Sorvall HB-4 rotor. Most of the mitochondrial and microsomal contaminants were removed by this step. The washed pellet was resuspended in 1 ml of 1.3 M sucrose-TKM gradient extending from 1.3 M to 2.0 M, and centrifuged at 130,000 g for 30 min in a Spinco 50L swinging-bucket rotor (27).

At the end of the run, microsomal contaminants were floating at the top of the aqueous solution, the cell-surface membrane ghosts and ghost fragments were layered in two bands closely spaced about one-fourth of the way down the gradient, and the nuclei were in a translucent pellet at the bottom of the tube (Fig. 6). The two bands were removed with a pipette and pooled. The remaining supernatant was discarded and the top of the nuclear pellet was rinsed with 0.25 M sucrose-TKM.

To remove adhering mitochondria and other contaminants, the plasma membrane preparation was diluted to a volume of 10 ml with 0.25 M sucrose-0.001 M ethylenediaminetetraacetic acid (EDTA) and homogenized with three strokes of a 10 ml Potter-Elvehjem glass-Teflon grinder at 1000 rpm. This homogenate was washed three times in 0.25 M sucrose-0.001 M EDTA at 1100 g for 10 min in the HB-4 rotor.

**Mitochondrial Fraction:** The 2000 g supernatant was centrifuged at 12,500 g for 25 min in a
instances. Washing consisted of resuspending the fractions (31).

Distinct bands in the gradient (Fig. 12) were removed from 1.04 to 4.5 ml continuous sucrose gradient (30) extending about 0.25 ml of this suspension was layered over a

adjusted to a concentration of -20 mg protein/ml.

was again loosely sedimented by centrifugation at

pellet were discarded. The microsomal suspension

remaining postmicrosomal supernatant and glycogen

suspended by pipetting in 0.25 ml of sucrose-TM and diluted to 4.5 ml with

M

M

and centrifuged for 6.5 hr at 200,000 g. Upon termination of the run, the four distinct bands in the gradient (Fig. 12) were removed separately with a J-shaped needle attached to a syringe (31).

Isolated fractions were either fixed for electron microscopy or washed to remove soluble matrix substances. Washing consisted of resuspending the fractions in distilled water, sonicating for 4 sec at the maximum intensity of a Bronwill Biosonik II sonicator at 0°C, and centrifuging at 100,000 g for 70 min in a Sorvall Type 30 rotor. These washed pellets of the fractions were precipitated with 10% (final concentration) trichloroacetic acid (TCA) and allowed to stand for a minimum of 2 hr at 4°C (8).

Radioactive Labeling

For incorporation and turnover studies, exponentially growing cells were labeled with glycerol-2-3H (500 µCi/µmole, New England Nuclear Corp., Boston, Mass.) at the concentrations specified in the various experiments. Other details of the treatment of cells during incorporation and turnover experiments have been described in the preceding paper (26).

For pulse-chase experiments, cells were suspended in fresh medium at a concentration of ~4 x 10^6 cells per milliliter and specified concentrations of glycerol-2-3H were added. After a 10 min pulse-labeling period, the cells were quickly harvested and washed twice by centrifugation at 125 g for 1.5 min. The wash period lasted ~3 min. The cells were then resuspended to a concentration of ~2 x 10^5 cells per milliliter in fresh medium containing 16.5 µmoles nonradioactive glycerol/ml.

Chemical and Radioactive Assays

Detailed explanations of the preparation of acid-soluble and -insoluble fractions of cells or cell fractions, as well as the technique for lipid extraction, have been given in the preceding paper (26). Total lipid phosphorus was determined as described by Marinetti (32). Protein was assayed according to Folin-Ciocalteau as modified by Miller (33), with bovine serum albumin as a standard. RNA was determined on washed TCA hydrolysates (34) by the Mejbaum orcinol procedure (35) with purified yeast RNA as a standard.

Microscopy

A Carl Zeiss-WL phase-contrast microscope was used for monitoring the fractionation procedure and for light micrographs. The approximate percentages of cell breakage after homogenization, as well as nuclear breakage in the nuclear pellet, were determined by direct counting.

Amebae were fixed in growth medium with 5 volumes of 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr at room temperature (36). Then the cells were gently centrifuged at 125 g for 2 min. Similar centrifugations were used at each subsequent step in fixation and dehydration. The cells were rinsed in five changes of cold buffer over a 2 hr period, postfixed in cold Zetterqvist's (37) osmium tetroxide solution for 45 min, rinsed in H2O, dehydrated through a graded series of ethanol solutions to propylene oxide, and embedded in Epon (38). In some experiments cells were not prefixed in glutaraldehyde.

Pellets from cell fractionations were resuspended in 0.5 ml of 0.25 M sucrose-TM and diluted to 4.5 ml with 2% glutaraldehyde in phosphate buffer. After 1 hr of fixation, the fractions were sedimented by centrifugation at 100,000 g for 40 min in a Sorvall 50L rotor, in 5-ml cellulose nitrate tubes, and processed thereafter as pellets in the centrifuge tubes. After postfixation in Zetterqvist's (37) osmium tetroxide solution and dehydration in alcohol, the bottom portions of the centrifuge tubes with the pellets were cut off with a razor blade and the cellulose nitrate was dissolved away in several changes in propylene oxide. The pellets were then cut in equal halves and embedded in Araldite, oriented so that the plane of thin sections included the entire thickness of the pellet.

Sections were cut on a Porter-Blum MT-1 ultramicrotome, placed on grids coated with 0.3% paraﬃn, and stained with 4% aqueous uranyl acetate and Reynolds' lead citrate (39). Specimens were examined with a Philips-300 electron microscope at 60 kV. The electron microscope was calibrated on the day of use with a carbon replica of a diffraction grating with 2160 lines per millimeter (Ernest F.
Fullam, Inc., Schenectady, N. Y.). Micrographs were taken at magnifications ranging from 3200 to 43,000 diameters. The dimensions of structures were measured directly on negatives with a Nikon Shadowgraph. At least 100 measurements of each structure were utilized to obtain the approximations reported below.

RESULTS

Cell Structure

The fine structure of *A. palestinensis* was found to be essentially identical to the published descriptions of related soil amebae (Neff's *Acanthamoeba castellanii* [36] and *Acanthamoeba* [= Hartmannella] *rhysodes* [40]). Therefore, only those structures pertinent to the present paper will be described.

There was a full complement of the membrane systems and membrane-bounded organelles usually found in eucaryotic cells (Figs. 1–4) [36]. These included RER, a small amount of SER, various Golgi-like membranes, plasma membranes, food vacuoles (a general term used in this paper to describe phagocytic or digestive vacuoles), a contractile vacuole, mitochondria, and a nucleus. Lipid droplets and glycogen were abundant (Fig. 5). In addition, a previously undescribed class of small, collapsed vesicles was observed throughout the cytoplasm. Their most frequent and characteristic form was cup-like, although discoidal vesicles of similar size were also observed (Fig. 1). The length of these structures varied from ~60 to ~370 nm, with an average length of 150 nm. In contrast to most other intracellular membranes, those composing the collapsed vesicles were identical in thickness (~100 Å) to plasma and food vacuole membranes and, like these membranes, were asymmetrical, with the leaflet adjacent to the cytoplasm being thinner or less densely stained than the other leaflet. Cells fixed for comparative purposes in Zetterqvist's (37) osmium tetroxide solution without prefixation in glutaraldehyde presented less aesthetic images (not pictured). Nonetheless, cup-like vesicles were still obvious, although more were distended to produce discoidal or spherical shapes.

Although collapsed vesicles were found throughout the cytoplasm, localized concentrations of these elements could be observed in association with Golgi-like cisternae (Figs. 1 and 4), food vacuoles (Fig. 2), and the cell surface (Figs. 4 and 5).

The membranes of food vacuoles were often thrown into folds, as noted by Bowers and Korn (36). In addition, the asymmetrical unit membrane was interrupted by furrows delimiting the edges of curved pieces, or plaques, with their concave surfaces facing the cytoplasm (Fig. 3). The average furrow-to-furrow distance, like the average length of collapsed vesicles, was ~150 nm. It was not uncommon to observe regions of food vacuoles where large numbers of collapsed vesicles seemed to have been fixed in the process of pinching off from and/or fusing with the limiting membrane of the vacuole (Fig. 2). In regions where groups of collapsed vesicles were near the cell surface, the plasma membrane was also rippled by furrows (Figs. 4 and 5). These regions of undulating plasma membrane were similar in structure to the food vacuole membranes described above (compare Figs. 3 and 5).

Morphological Characterization of Cell Fractions

Because the goal of the present research was to correlate kinetic data obtained from cell fractions with cytological elements in intact cells, preservation of these structures was the main criterion guiding the development of the cell fractionation procedure (see Methods section). Two physical factors seemed to be of primary importance in this regard. (a) Gentle homogenization: cells were homogenized to an extent which gave maximum cell rupture (~90%) and very little (~10%) nuclear damage as judged by phase-contrast microscopy. For this reason, cytoplasmic structures smaller than nuclei were not expected to be severely damaged. (b) Gentle centrifugation: both the nuclear-plasma membrane pellet (2000 g) and the microsomal pellet (100,000 g) were sedimented at forces and over time intervals adjusted to yield loose, as opposed to compact, pellets. This practice had the benefit of allowing easy resuspension by pipetting and little loss of microsomal membranes in the underlying glycogen. The homogenization required to resuspend compact pellets was found to break up membranous elements into very small vesicles (cf., 41, 42).

| PLASMA MEMBRANE FRACTION: After subfractionation of the 2000 g pellet on a 1.3 M–2.0 M sucrose-TKM gradient the cell surface ghosts and their fragments formed two bands closely layered about one-fourth of the way down the |
gradient (Fig. 6). The components of the two bands appeared identical as visualized with the electron microscope. Perhaps the formation of two bands was a cation effect (43, 44). Homogenization and washing of the ghosts at 1000 g for 10 min in 0.25 M sucrose-0.001 M EDTA removed mitochondrial contaminants and caused some vesiculation of the plasma membranes (Fig. 7). Most of the membranes were curved, with the thicker leaflet on the convex side, just as in intact cells (compare Figs. 5 and 8). Only plasma membrane-like elements were visible in this fraction by electron microscopy. As further indication of the lack of cytoplasmic contamination, no RNA was detectable chemically (35). Food vacuole membranes, morphologically indistinguishable from plasma membranes (45), may have contaminated this fraction, but evidence will be presented below that food vacuoles were recovered in the mitochondrial fraction.

**Nuclear Fraction:** When viewed with a phase-contrast microscope the nuclear pellet (Fig. 6) appeared homogeneous with few (~10%) broken nuclei (Fig. 9). Electron micrographs illustrated that most of the remnants of broken nuclei, as well as plasma membrane and mitochondrial contaminants, were in the upper portion of the nuclear pellet (Fig. 10). For this reason, the top of the translucent nuclear pellet (Fig. 6) was rinsed with 0.25 M sucrose-TKM before processing for chemical or radioactive studies (see Methods section). No cytoplasmic RER was seen adhering to the outer, rough-surfaced nuclear membrane. In some cases, portions of the outer membrane had been sheared off (Fig. 11).

**Mitochondrial Fraction:** The 12,500 g pellet contained mitochondria, cell surface membrane fragments, RER, and large numbers of vacuoles containing material similar to that seen in food vacuoles in intact amebae (not pictured). The heterogeneity of the mitochondrial fraction precluded its use in this study.

**Microsomal Fraction:** After subfractionation of the 100,000 g pellet on a continuous sucrose gradient, four distinct bands were visible (Fig. 12).

Band 1, a narrow band at the top of the gradient, was composed almost entirely of collapsed vesicles (Figs. 13 and 14). The pellet of the band was stratified so that larger vesicles appeared in the middle and bottom portions (Fig. 13) while smaller vesicles were on top (Fig. 14). The fact that this subfractionation accounted for ~52% of the lipid phosphorus of the microsomes (Table I) was not surprising in view of the almost ubiquitous distribution of these vesicles in fixed cells (Figs. 1–5). Some of the vesicles might have been produced by fragmentation of the food vacuole and plasma membranes. However, as described above, plasma membranes were not observed to vesiculate until their final washings in 0.25 M sucrose-0.001 M EDTA. A very small amount of SER was detectable in the bottom portion of the pellet. Although contamination with RER was not apparent, small particles similar in dimensions to ribosomes were occasionally observed. However, the average RNA/protein ratio of this fraction was only 0.04 (µg/µg).

Band 2, a narrow band about one-fourth of the way down the gradient, was enriched with mem-

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**Figure 1** An electron micrograph of a section through the cytoplasm of *A. palestinensis* illustrating collapsed vesicles (C) in association with Golgi-like cisternae (G). The cup-shaped vesicles appear crescentic when sectioned longitudinally (C1) and as concentric circles when cut transversely (C2). When cut normally, the unit membranes of these vesicles appear asymmetrical, thicker or more densely stained on the luminal side, and similar in thickness and asymmetry to those of the Golgi-like cisternae with dilated ends (G). A lipid droplet (L), a mitochondrion (m), and densely stained glycogen particles can be observed in this image. × 70,000.

**Figure 2** An electron micrograph of a food vacuole (FV). Disc-shaped collapsed vesicles are found in large numbers throughout the cytoplasm surrounding the food vacuole, which is distinguished by its asymmetrical unit membrane and its content of amorphous material (33). In some areas, collapsed vesicles appear to be pinching off from and/or fusing with the limiting membrane of the food vacuole (arrow). × 28,000.

**Figure 3** The limiting membrane of this food vacuole (FV) appears to be composed of curved plaques whose edges are delimited by furrows (arrowheads). × 50,000.
branous structures which were identical to the Golgi-like cisternae previously described in fixed cells (compare Figs. 1, 4, 15, and 16). When their asymmetric membranes were cut in profile, the cisternal structures revealed distended ends, often containing a dense matrix and/or membranous structures (Fig. 16). The entire thickness of the Golgi-enriched fraction contained collapsed vesi-

**FIGURE 4** An electron micrograph of a section through the cellular periphery. Concentrations of collapsed vesicles (arrows) are observed underlying the plasma membrane (PM), which even at this magnification seems composed of undulating segments of membrane. The central group of vesicles is associated with a Golgi complex (G). **(rer):** rough endoplasmic reticulum. \( \times 30,000 \).

**FIGURE 5** This portion of plasma membrane appears to be composed of plaque-like structures separated by furrows (arrows). **(C):** collapsed vesicle. \( \times 100,000 \).
cles and the middle and lower portions of the pellet included RER. This fraction comprised about 4% of the lipid phosphorus of the total microsomes (Table I) and had an average RNA/protein ratio of 0.09.

Band 3, a narrow band in the middle of the gradient, contained a mixture of the Golgi-like elements described above, RER, and vesicular and tubular SER (not pictured). Approximately 14% of the microsomal lipid phosphorus was present in this fraction (Table I) which had an average RNA/protein ratio of 0.15. Due to the heterogeneous nature of the fraction, it was not utilized in these experiments.

Band 4 was a broad band in the lower one-half of the gradient and contained RER (Figs. 17 and 18). Occasionally, collapsed vesicles were observed. Some of the RER vesicles contained a matrix. The pellet of this band was stratified so that the upper portion contained primarily cisternal structures (Fig. 18) while the middle and lower portions contained both cisternal and vesicular structures (Fig. 17). This fraction contained ~30% of the microsomal lipid phosphorus and had an average RNA/protein ratio of 0.25.

**Kinetics of Incorporation and Short-Term Turnover of Glycerol-3H-Labeled Phospholipids in Cell Fractions**

The preceding paper (26) established that most (~96%) of the glycerol-3H incorporated into total microsomal lipids was in phospholipids. For this reason, it was deemed unnecessary to separate the phospholipids from the total lipid extracts (chloroform:methanol) of the cell fractions.

![Figure 6](image-url)

*FIGURE 6* A diagrammatic representation of the disposition of subfractions of the 2000 g pellet after centrifugation at 190,000 g for 30 min in a sucrose-TKM gradient.

![Figures 7-8](image-url)

*Figures 7-8* Electron micrographs of the plasma membrane fraction. Fig. 7: At low magnification most of the membranes appear to be in the form of long pieces, although vesicles of varying sizes are observed. × 10,500. Fig. 8: At higher magnifications the unit membranes appear to be identical to plasma membranes in situ. The membranes are usually curved so that the thicker leaflet is outermost. × 100,000.
The early stages of incorporation of glycerol-\(^3\)H into the phospholipids of cell fractions followed a characteristic and repeatable pattern (Fig. 19, one of two identical experiments). Incorporation appeared to be most rapid into phospholipids of the nuclei and RER and slowest into phospholipids of the Golgi membranes, collapsed vesicles and plasma membrane fractions, in that order (i.e., nuclei and RER > Golgi membranes > collapsed vesicles > plasma membrane).

This pattern was maintained during at least 2 hr of incorporation (Fig. 20, one of three identical

**Figures 9-11** Phase-contrast (Fig. 9) and electron micrographs (Figs. 10-11), of the nuclear fraction. Fig. 9: Phase-contrast monitoring of the nuclei recovered after gradient centrifugation revealed few broken nuclei and little cytoplasmic contamination. ×1000. Fig. 10: The upper portion of the nuclear pellet contains most of the broken nuclei and cytoplasmic contaminants of this fraction, such as plasma membrane fragments (PM) and mitochondria (m). ×5500. Fig. 11: Approximately 40% of the nuclei are missing portions of the outer, rough-surfaced membrane (arrowheads). Note also the nuclear pores in tangentially sectioned areas of the nuclear membranes. ×20,000.
experiments). However, in the interval between 2 and 10 hr, the smooth membrane fractions (Golgi membranes, collapsed vesicles, and plasma membrane fractions) achieved rates of incorporation equivalent to those of the rough membrane fractions (nuclei and RER). The reversal in relative position between plasma membranes and collapsed vesicles in Fig. 20 was found repeatedly. Thus, in late stages of incorporation the positions of the fractions in terms of specific activities were:
nuclei and RER > Golgi membranes > plasma membranes > collapsed vesicles.

In the above experiments the isolated fractions had been sonicated briefly and washed before lipid extraction. However, it also was found that unwashed cell fractions, as seen in Figs. 7–11 and 13–18, had specific activities identical to those of the washed fractions. Apparently little, if any, of the new radioactive phospholipid of the fractions was concentrated in the form of a soluble matrix, either in the intracisternal spaces of the various membranes or in the nucleoplasm.

When cells labeled for 12 hr were washed and resuspended in chase medium containing nonradioactive glycerol (Fig. 21, one of two identical experiments), all of the specific activities of the membrane fractions decreased over a 24 hr period at a rate greater than that expected from dilution by growth, an indication of some turnover of membrane phospholipids. The nuclei and RER consistently lost specific activity more rapidly than the smooth membrane fractions, and the positions of the specific activities of the smooth membrane fractions were reversed (collapsed vesicles > plasma membranes > Golgi membranes > nuclei and RER).

**Relative Specific Activities during Incorporation and Turnover**

In order to pool the results of the several experiments, the specific activity of each fraction at each time point was converted to a specific activity ratio relative to the activity of the nuclear fraction (Fig. 22). The nuclear fraction was chosen arbitrarily because it maintained the highest specific activity throughout incorporation.

The virtually complete parallelism between the specific activity ratios of nuclei and RER throughout incorporation and turnover is obvious, and emphasizes the similarity in their rates of incorporation and turnover of phospholipids. The constant difference between the ratios of nuclei and RER presumably was primarily due to differing lipid phosphorous compositions of the two

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**Table I**

*Distribution of Lipid P in Subfractions of Microsomes*

| Subfraction         | µg Lipid P/Subfraction* |
|---------------------|-------------------------|
|                     | Expt. 1 | Expt. 2 | Expt. 3 | Average |
| Band 1 (collapsed vesicles) | 17.50 (55) | 18.90 (54) | 15.25 (46) | 17.90 (52) |
| Band 2 (Golgi)      | 1.17 (4) | 1.30 (4) | 1.02 (3) | 1.15 (4) |
| Band 3 (mixture)    | 4.70 (15) | 4.95 (14) | 4.25 (13) | 4.65 (14) |
| Band 4 (RER)        | 6.86 (26) | 10.00 (26) | 12.62 (38) | 9.47 (27) |
| Total               | 31.72 (100) | 35.15 (100) | 33.14 (100) | 33.14 (100) |

* Lipid determinations were carried out on the subfractions of the total microsomes recovered from ~3 ml of wet, packed cells.
fractions. The differing lipid phosphorus compositions could have been due to the more or less constant contamination by other membranes in these fractions (see above).

In contrast, early in incorporation (0–2 hr), the smooth membrane fractions always exhibited a lag in incorporation relative to the nuclear and RER fractions. However, by 10 hr after the start of incorporation, the ratios of the smooth membrane fractions had approached those of the rough membrane fractions and were maintained until the cells were placed in chase medium at 12 hr. The stable positions of the ratios, seen as horizontal lines between the 10- and 12-hr points in Fig. 22, indicate identical rates of incorporation of radioactive phospholipids into the different membranes. While horizontal lines describe the stability of the ratios, the specific activities of the membranes were actually increasing during this time (see Fig. 20). The previously noted change in position of the ratio of the plasma membrane between early and late stages of incorporation can be seen.

During turnover experiments, the ratios of the smooth membrane fractions were reversed relative to each other and to the nuclei and RER within 20 hr (32 hr point on Fig. 22) after the cells were placed in chase medium. Like the positions of the ratios attained after prolonged incorporation, the positions of these turnover ratios were stable and indicative of identical rates of turnover in the membranes (compare to Fig. 21).

**Kinetics of Pulse-Chase Labeling**

The rapidity with which glycerol-3H is incorporated into cellular lipids in *A. palestinensis* (26) makes it an appropriate label for pulse-chase experiments. During the chase period after a 10 min labeling period and a 5 min washing period (Fig. 23) the acid-soluble intracellular pool of radioactivity remained small and there was found to be little or no net synthesis of acid-insoluble lipid. Therefore, any increase in the specific activity of a fraction during the chase period can be interpreted as being due to the incorporation of presynthesized phospholipid.

In fractions isolated from cells 45 min after a pulse label, nuclei, RER, and Golgi membranes had decreased in specific activity, whereas plasma membranes had continued to increase rapidly in specific activity and collapsed vesicles had increased at a slower rate (Fig. 24, one of two identical experiments). In the interval between 60 and 120 min after the start of the experiment, the plasma membrane fraction decreased in specific activity, while other fractions changed little (Fig. 24).

**DISCUSSION**

The cell fractions utilized in these experiments were relatively homogeneous as judged by electron microscopy (Figs. 7–11, 13–18) and the lipid radioactivity measurements obtained from them were assumed to reflect events occurring in the membranes. The differing lipid phosphorus compositions could have been due to the more or less constant contamination by other membranes in these fractions (see above).

In contrast, early in incorporation (0–2 hr), the smooth membrane fractions always exhibited a lag in incorporation relative to the nuclear and RER fractions. However, by 10 hr after the start of incorporation, the ratios of the smooth membrane fractions had approached those of the rough membrane fractions and were maintained until the cells were placed in chase medium at 12 hr. The stable positions of the ratios, seen as horizontal lines between the 10- and 12-hr points in Fig. 22, indicate identical rates of incorporation of radioactive phospholipids into the different membranes. While horizontal lines describe the stability of the ratios, the specific activities of the membranes were actually increasing during this time (see Fig. 20). The previously noted change in position of the ratio of the plasma membrane between early and late stages of incorporation can be seen.

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Figures 19-20 Appearance of glycerol-$^3$H-labeled phospholipids in cell fractions during incorporation. Fig. 19: 0-15 min. Exponentially growing cells were labeled in medium containing 40 µCi glycerol-$^3$H/ml (80 nmoles/ml). The symbols are: •, nuclei; ■, plasma membrane; △, collapsed vesicles (band 1); ▲, Golgi-like fraction (band 2); ○, RER (band 4). Fig. 20: 0-12 hr. Cells were labeled in medium containing 20 µCi glycerol-$^3$H/ml (40 nmoles/ml). Symbols are given in Fig. 19.

Figure 21 Turnover of glycerol-$^3$H-labeled phospholipids in cell fractions. Cells were labeled for 12 hr in medium containing 2 µCi glycerol-$^3$H/ml (4 nmoles/ml). After this period the cells were washed twice and resuspended in chase medium containing 10.5 μmoles nonradioactive glycerol/ml, beginning at time 0. The dashed line represents the expected growth dilution of the specific activity of the nuclear fraction. Such growth dilution rates for fractions beginning with lower initial activities would have even shallower slopes. All fractions exceeded their expected rates of dilution by growth. The other symbols are given in Fig. 19.

Radioactive phospholipids were incorporated preferentially into the nuclear and RER fractions during the early stages of incorporation, while a lag in incorporation was evident in other membranes (Fig. 19). Radioactively labeled phospholipids reportedly are synthesized in the endoplasmic reticulum of adult rat liver cells (9, 10) and were found to be incorporated initially into the RER of developing rat hepatocytes (8). In A. palestinensis, neither RER nor nuclear fractions lost label upon washing; possibly the newly synthesized phospholipid was integral with the membranes of these fractions. This would be consistent with morphologically similar elements of fixed cells (Figs. 1-5). Because the cells were growing and doubling over a 27 hr period, we were, in effect, studying the phospholipids of growing membranes.

Some turnover of phospholipids occurred in all of the cellular membranes measured (Fig. 21). This is consistent with our previously reported suggestion (25) that some membrane phospholipids are degraded into neutral lipids and transferred to lipid droplets.

The specific activity of each fraction is expressed as the ratio: (specific activity of fraction)/(specific activity of nuclei). Each point on this graph is the average of one to four experiments (15 min, two experiments; 2 hr, two experiments; 10 hr, one experiment; 18 hr, four experiments; 38 hr, one experiment; 56 hr, two experiments). Symbols are given in Fig. 19.
RER are morphologically similar, topologically continuous (47, 48, 49), and have similar enzymatic activities (47, 50). Therefore, it would not be surprising if they shared the function of synthesizing membrane phospholipids. In any case, RER and nuclei are considered as a functional unit in this discussion.

Data from pulse-chase experiments (Fig. 24) indicate a rapid turnover of glycerol-3H in newly synthesized phospholipid in nuclei and RER which, together with the evidence for rapid synthesis there, can be explained only by rapid destruction or by export of newly formed phospholipid to other parts of the cell. The Golgi fraction showed less rapid turnover, while plasma membranes and collapsed vesicles continued to increase in specific activity during the first 45 minutes of chase, at a time when the only apparent source of label was presynthesized phospholipid. If these data indicate migration of labeled phospholipids, a likelihood discussed below, then the order, based upon relative rates of either early incorporation or acute turnover, would be nuclei and RER → Golgi membranes → collapsed vesicles → plasma membrane. During the second hour of chase (a small fraction of the cell cycle) only the plasma membrane decreased in specific activity, perhaps indicating that some of the newly synthesized phospholipid had become a relatively stable component of the other membranes.

During both prolonged incorporation and prolonged chase, periods of relative equilibrium (Fig. 22), the order of apparent replacement of phospholipid was slightly, but consistently, different from that found in acute experiments; it became nuclei and RER → Golgi membranes → plasma membrane → collapsed vesicles. If the data from acute experiments indicate the migration of newly formed phospholipids, then the more slowly reached stable specific activity ratios must indicate a more complex pattern of movement.

The various membranes of rat liver have different phospholipid compositions (51) and this probably holds true in *A. palestinensis*. Therefore, any interpretation of the kinetic results obtained from the glycerol-3H labeling experiments in *A. palestinensis* must take into account the fact that the rates of synthesis, turnover, and reutilization of the glycerol-3H moiety of the various phospholipids are not known. The half-life of glycerol-3H in phospholipids of *A. palestinensis* could be similar to the ~40 hr half-life of the glycerol moiety ob-

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**Figure 23** Pulse-labeled acid-soluble (△—△) and acid-insoluble (●—●) materials of cells. Cells were exposed to 12.5 µCi glycerol-3H/ml medium (25 nmoles/ml) for a 10 min pulse period, washed twice over the next 5 min, and resuspended in chase medium containing 16.5 nmoles nonradioactive glycerol/ml.

**Figure 24** Pulse-labeled cell fractions. Cells were pulse-labeled with 20 µCi glycerol-3H/ml medium (40 nmoles/ml), washed, and chased as described in Fig. 23. Symbols are given in Fig. 19.
served in liver membrane phospholipids (4, 52), or conversely, the situation in growing A. palestinesis could be analogous to that of growing mouse-L-cells, where little or no apparent turnover of membrane components has been reported (53). A slow turnover of the labeled phospholipids, with or without reutilization of the glycerol-3H moiety, should have little influence in acute experiments (Figs. 19, 23, and 24). Therefore, it seems reasonable to interpret these acute results as due to migration of phospholipids.

On the other hand, reutilization of glycerol-3H for phospholipid synthesis would probably affect the results of the longer incubations used in some of our experiments (Figs. 20 and 21). Such reutilization should be confined to the locus of synthesis of phospholipids, the nucleus and RER, and might account for the relative equilibrium of rates of incorporation and turnover attained during prolonged experiments (Fig. 22). Our data do not allow us to decide whether or not this is the case.

Further analysis of the slowly reached, stable specific activity ratios observed during both prolonged incorporation and prolonged chase must include a consideration of whether phospholipids are relatively stable components of membranes or whether an exchange of phospholipids occurs between membranes, possibilities which are best discussed in the context of the mode of transport of phospholipids from their site of synthesis in the RER and nuclei to other cellular membranes. The mechanisms by which phospholipids synthesized in one type of membrane are transposed to other types of membranes, occupy strategic positions in current theories of membrane biosynthesis. Two major hypotheses have been used to explain the origin of cellular membranes: (a) All the different cellular membranes grow by self-assembly. That is, soluble macromolecular complexes or individual molecules attach to membranes during their growth or as replacements for degenerate portions of those membranes (2, 3, 4, 5, 6, 7); (b) a primary membrane composed of phospholipids and perhaps structural protein (54) is assembled at a central site and transported to places where various other cellular membranes are needed (8). During transportation an orderly conversion could be effected by the selective addition and deletion of those molecules that make each type of membrane morphologically unique and functionally different.

To date, most studies on membrane proteins argue against the concept of a long-lived primary membrane composed of proteins and phospholipids. It has been reported that the different membranes of liver have diverse protein compositions (55, 56). Presumably, this is a reflection of the functional diversity of the various cellular membranes and would probably be applicable to A. palestinesis. Examinations of the kinetics of total membrane protein synthesis and deposition using radioactive amino acid labels have shed little light on the problem of membrane synthesis (4, 52), whereas studies concerned with specific membrane proteins have yielded information about the insertion (50), turnover (4), and reutilization (57, 58) of protein moieties. It now seems that most of the proteins of different membranes of liver are turning over at different rates (4, 57, 58). The presence or absence of a structural protein common to all cellular membranes is still in dispute (54, 55, 56, 57). Thus, observations on membrane proteins, while yielding information on those constituents that make membranes unique, do not rule out the possibility that the phospholipid components and perhaps structural proteins of some cellular membranes are assembled as membranes at a locale distant from their eventual site of utilization.

There is compelling evidence that phospholipids synthesized in the endoplasmic reticulum of liver are transported to the membranes of mitochondria via a soluble route by a carrier-protein-mediated exchange of phospholipids (59, 60, 61). In the present experiments, although no large soluble pool of phospholipids was demonstrable, a rapid shuttling of soluble phospholipids to self-assembling membranes is possible. However, the existence of an ordered sequence in the appearance of label in the phospholipids of the various membranes (Figs. 19 and 24) indicates that any flow of soluble phospholipids from the RER and nuclear membranes to other cellular membranes is probably not a simple diffusion process. In many cells associations between the RER and the SER-like membranes of the "forming face" of the Golgi complex have been observed (22, 25, 62, 63, 64, 65, 66, 67) and there is evidence that newly synthesized proteins and lipoprotein granules are transported through the intracisternal elements of the endoplasmic reticulum to the Golgi cisternae (21, 68). A similar intracisternal flow of newly synthesized phospholipids to Golgi membranes...
could provide a "directed" transport of soluble phospholipids in *Acanthamoeba*.

Alternatively, it is also possible that a transfer of phospholipids by membrane transconversion occurs. The idea that membranes originally derived from the RER move through the Golgi complex from a forming face to a "mature" face is not new and is well-founded in morphological observation (22, 25, 62, 63, 64, 65, 66, 67). In fact, the regeneration of the Golgi complex has been shown to be dependent upon the nucleus in *Amoeba proteus* (67). Furthermore, cytoplasmic RER was associated and often continuous with the forming face of regenerating Golgi cisternae in *Amoeba proteus*. Our data are not unequivocal on these points and do not allow us to distinguish between a "directed" flow of soluble phospholipids, membrane transconversion, or some less obvious mechanism.

In *A. palestinensis*, the size distribution of curved, plaque-like structures composing portions of the plasma and food vacuole membranes was found to be similar to the size range (60–370 nm) of the length of collapsed vesicles observed throughout the cytoplasm. Furthermore, concentrations of collapsed vesicles were frequently observed underlying the plasma membrane, pinching off from and/or adding to food vacuole membranes, and in association with Golgi-like cisternae. This association between vesicles with asymmetrical membranes and similar plaques in the cell surface is reminiscent of the discoidal vesicles and specialized plasma membranes of transitional epithelial cells in mammalian urinary bladder (69). Like collapsed vesicles in *A. palestinensis*, discoidal vesicles of urinary bladder have closely apposed membranes and little intracisternal space, which would seem to render both structures inefficient for transport of substances. Discoidal vesicles in transitional epithelium can pinch off from the plasma membrane, as demonstrated by their uptake of ferritin from the lumen of the bladder, and it has been proposed that they also originate from the Golgi apparatus as a source of new plasma membrane (70, 71). Thus, there is evidence from both transitional epithelium and *A. palestinensis* to suggest that specialized vesicles form a mobile pool of preformed plasma membrane. By morphological and radioautographic experiments on a variety of cell types, vesicles derived from the Golgi complex also have been implicated as vectors involved in the transport of extracellular coat or scale materials to the cell surface (56, 72, 73, 74, 75, 76).

It has been shown by Bowers (16) and Korn and Weisman (18, 19) that phagocytic and pinocytic vesicles in *Acanthamoeba castellanii* (Neff) are derived from plasma membrane. Once internalized, these vesicles fuse with one another and eventually become digestive vacuoles. Large amounts of plasma membrane are rapidly and constantly internalized in this manner by growing cells (16, 17, 18); therefore, a mechanism must exist for degrading or reutilizing such membrane. If food vacuole membranes in *A. palestinensis*, after serving their nutritive purpose, were dismantled by pinching off of collapsed vesicles, the resulting dilution in specific activity of the pool of collapsed vesicles could account for the altered sequence: nuclei and RER \(\rightarrow\) Golgi membranes \(\rightarrow\) plasma membrane \(\rightarrow\) collapsed vesicles observed in later stages of incorporation, as well as following a prolonged period of turnover (Fig. 22). In *A. castellanii*, phagocytosis of polystyrene latex beads essentially stopped after a few hours, leaving the undigestible plasma membrane-bounded beads entrapped in the cytoplasm (45). Possibly this cessation of phagocytosis was due to the exhaustion of the supply of plasma membrane which normally would have been replaced by recycling of phagocytic membrane through the pool of collapsed vesicles. Consistent with this, the synthesis of new membrane phospholipids, as measured by incorporation of radioactively labeled phospholipid precursors, was reported to be unaffected by the phagocytosis of latex beads (77). Presumably, the normal production of new plasma membrane continued, but was not rapid enough to allow any significant amount of phagocytosis in these overloaded cells.

In conclusion, it would appear that the RER and nuclear membranes are the source of phospholipids for all the membranes examined in *A. palestinensis*. In addition, a sequence in the glycerol-\(^{1}H\) labeling of the phospholipids of the various membranes exists. Whether this sequence is due to (a) transconversion of a primary phospholipid membrane, (b) rapid and directed flow of soluble phospholipids, (c) a combination of these two events, or (d) some other mechanism cannot be unequivocally decided with the present data. However, the combined morphological and kinetic experiments do allow us to postulate that the
plasma membrane is derived from and maintained by a recycling pool of cytoplasmic vesicles.

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