ISOLATION OF THE MEMBRANE SYSTEMS OF *ACANTHAMOEBA PALESTINENSIS*: STUDIES OF LIPID SYNTHESIS AND ASSEMBLY OF LIPIDS INTO MEMBRANES

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ABSTRACT

ISOLATION OF THE MEMBRANE SYSTEMS OF *ACANTHAMOEBA PALESTINENSIS*: STUDIES OF LIPID SYNTHESIS AND ASSEMBLY OF LIPIDS INTO MEMBRANES

By

Francis Joseph Chlapowski

A fractionation procedure is described for the isolation of membranes and membrane-bound organelles of *Acanthamoeba palestinensis*. Nuclei, mitochondria, rough endoplasmic reticulum, elongate smooth endoplasmic reticulum, small cisternal smooth membranes, Glogi membranes and a fraction containing plasma and digestive vacuole membranes were isolated using this procedure. Electron micrographs, chemical analysis and enzyme assays were used to characterize these fractions. Utilizing C^{14}-choline as a specific marker of phosphatidyl choline, it was demonstrated that an exchange reaction occurred between free choline and the choline moiety of phosphatidyl choline. This exchange reaction occurred only on membranes in a cell free system. A time sequence study of H^{3}-glycerol incorporation, turnover and intracellular movements in lipids is reported. Most lipids are synthesized in the rough endoplasmic reticulum and transferred to the non-membrane lipids recovered in the post-microsomal supernatant. The nuclear membranes and rough endoplasmic reticulum are implicated as sites of lipid assembly into membranes. Cyclical patterns in the pulse and chase studies indicated a recycling of lipids among the different membrane types. These results suggest the possible recycling of membranes, involving interconversions among membranes.

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By

Francis Joseph Chlapowski

A THESIS

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To

Dad and Mom,

Rev. Henry S. Banach

and

Susan

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This thesis would not have been possible, if it were not for the untiring work and consideration of Dr. R. Neal Band on my behalf. I shall always remember and appreciate his original and dynamic manner, which has often served as a guide in my own approach to scientific problems. Thank you for everything Dr. Band.

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INTRODUCTION

In the evolution from procaryotic cells to eucaryotic cells, membrane development plays a singularly important role. In the smallest unit capable of genetic continuity - the virus - no membrane structures are present, with the exception of the myxovirus, whose envelope seems to be derived from the cell surface membrane of the infected cell (Davis, Dulbecco, Eisen, Ginsberg and Wood, 1967). Most procaryotes have only a cytoplasmic membrane underlying the cell wall. Some Gram-positive bacteria contain mesosomes. These invaginations of the cytoplasmic membrane might be involved in respiratory functions, similar to the mitochondria of eucaryotic cells. Whereas procaryotic cells are essentially devoid of membranous components, eucaryotic cells contain a complex array of membranes and membrane-bound entities. A general higher cell type is encompassed by a plasmalemma and is composed of a double-membrane nuclear envelope, granular endoplasmic reticulum, smooth endoplasmic reticulum, Golgi-complex membranes, mitochondria constructed of inner and outer membranes and various vesicular components (e.g. lysosomes, peroxisomes, pinocytotic or phagocytotic vacuoles, etc.). Photosynthetic eucaryotic cells also contain chloroplasts surrounded by two membranes. Thus, more intricate functions at the cellular level are accompanied by an amplification of the membrane systems.

The Danielli-Davson model (1935) suggests that cell membranes are composed of a bimolecular layer of lipid covered with a monomolecular layer of protein on each side. Electron micrographs (Robertson, 1959),

as well as X-ray diffraction patterns (Finean, 1953; Finean, Coleman, Green and Limbrick, 1966), have been interpreted as support for this concept. The ultrastructural resemblance among membranes derived from different sources has led to the term "unit membrane" (Robertson, 1960). The unit membrane theory suggests that all membranes are lamellar in construction due to the predictions of the Danielli-Davson model. Korn (1960) has recently criticized the unit membrane theory as lacking in proof (see also Curtis, 1967; Weiss, 1967). An alternative to the lamellar composition proposed for membranes might be micelles of a hexagonal character (Luzzati and Husson, 1962). Room temperature negative staining of plasma membranes has illustrated such properties (Benedetti and Emmelot, 1965). It is not known whether the observed micelles are an intrinsic property of the membrane or a fixation artifact. Green and Perdue (1966) reject the Danielli-Davson model, as well as the unit membrane theory, and emphasize that the basic framework of membranes is protein. These authors propose that membranes are basically repeating units of lipoproteins joined by hydrophobic bonds. One of the principal reasons for their contention is the fact that mitochondrial membranes retain their ultrastructural characteristics after severe lipid extraction. This is also true in myelin and other membranes (Napolitano, LeBaron and Scaletti, 1967). However, the most important reason put forth by Green and his colleagues for the emphasis on protein in membrane systems is their isolation of a structural protein from mitochondrial membranes (Green, Tisdale, Criddle, Chen and Bock, 1961; see also Green, Haard, Lenaz and Silman, 1968). Recently, Woodward, Kubic, Kleese and Woodward (1968) have isolated

electrophoretically similar structural proteins from membranes of Neurospora mitochondria, corn mitochondria and corn chloroplasts. This finding establishes the notion of a membrane structural protein more firmly. Newer concepts of membrane structure and function place more emphasis on the role of proteins, macromolecular complexes and hydrophobic bonding. Less emphasis is placed on the role of electrostatic attractions between polar groups of lipids and proteins. However, independent of the importance of proteins in the structure of membranes, all biological membranes of eucaryotes are known to contain phospholipids and sterols, although the specific amounts of each may vary from one source to another (Ashworth and Green, 1966; Finean, 1967; Korn, 1966; Takeachi and Terayama, 1965).

The principal impediment in analyzing the membrane systems of cells is the isolation of these systems from the cell. In the case of membrane-bound organelles, the problem is simplified. The isolation of the organelle separates its membranes from other cellular membranes. One of the first cellular organelles isolated was the nucleus (Dounce, 1943; Allfrey, Stern, Mirsky and Saetren, 1952). More recent advances have yielded nuclear preparations of high purity in aqueous media (Chaveau, Moule and Rouiller, 1956; Maggio, Siekevitz and Palade, 1963; Blobel and Potter, 1966). Widnell and Siekevitz (1967) have reported a method for obtaining preparations of nuclear membranes from rat liver nuclei. Many methods have been developed for the isolation of mitochondria (Schneider, 1947; Hogeboom, Schneider and Palade, 1948). Caplan and Greenawalt (1966, 1968) have succeeded in separating the inner and outer membranes of mitochondria. The isolation of lysosomes and

peroxisomes has been accomplished by the work of deDuve and his collaborators (deDuve, Pressman, Gianetto, Wattiaux and Appelmans, 1955; Leighton, Poole, Beaufay, Baudhuin, Coffey, Fowler and deDuve, 1968). In animal cells the isolation of Golgi material has essentially been restricted to cells in which the Golgi system is extensively elaborated, such as the rat epididymis (Schneider, Dalton, Kuff and Felix, 1953; Schneider and Kuff, 1954). An exception to this has been the recent successful isolation of the Golgi apparatus from rat liver cells (Morrè, Mollenhauer, Hamilton, Mahley and Cunningham, 1968). Microsomes are generally isolated by high speed centrifugation, with subsequent density gradient fractionation (Britten and Roberts, 1960) to resolve granular and agranular microsomes (Palade, 1955; Palade and Siekevitz, 1955; Rothschild, 1961; Ernster, Siekevitz and Palade, 1962; Dallner and Nilsson, 1966; Dallner and Ernster, 1968). With the exception of red blood cell ghosts (Ponder, 1955), the most difficult cell membrane to isolate is the cell surface membrane. Neville (1960) first isolated the cell surface membranes of rat liver cells. Wallach and Kamat (1964) claim to have isolated the plasma membranes of Ehrlich ascites carcinoma cells (see also Kamat and Wallach, 1965; Wallach, 1967). The methods of Warren, Glick and Mass (1967) have been used to isolate fixed cell surface ghosts from mouse L cells. The isolation of a biologically active plasma membrane ghost preparation from HeLa cells has been described by Bosmann, Hagopian and Eylar (1968).

The biosynthesis of lipids and proteins, the enzymatic properties of membranes (e.g. Ernster, Siekevitz and Palade, 1962; Novikoff, Essner, Goldfischer and Heus, 1962), the intracellular movements of membranes

(e.g. Friend and Farquhar, 1967) and the role of membranes in transport, storage and secretion (e.g. Jamieson and Palade, 1967a, 1967b, 1968a, 1968b; Bowers and Korn, 1967; Beams and Kessel, 1968) are areas under intensive investigation. Although the literature is replete with hypotheses and research concerned with membrane biosynthesis and degradation, the actual events in the process by which the molecular components of membranes are united into a macromolecular complex can not be defined at this time. Since different membrane systems are quite similar with respect to gross chemical composition, but operate in different structural and functional situations, the assembly of membranes is a fascinating area of research. Because of the fundamental resemblance of various cellular membranes, the origin of one type of membrane by modification of a pre-existing, but different, type, is not an unreasonable thought. In fact, some evidence has been assembled which shows the fusion of plasma membranes, or their derivatives, with membranes derived from the Golgi complex (e.g. Friend and Farquhar, 1967; Bowers and Korn, 1967). Considering all the latter facts, an investigation into the movement of radioactive precursors into and out of the phospholipid molecules of membranes might be expected to shed some light on the biogenesis of membranes.

The organism chosen for this research was the soil amoeba Acanthamoeba palestinensis. Ultrastructural evidence will be given to demonstrate that Acanthamoeba palestinensis represents a typical eucaryotic cell type containing a full complement of membranous organelles. For this reason, and because growth of homogeneous populations under controlled axenic conditions is possible, this organism is

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particularly suitable for the investigation of membrane systems at the cellular level. A prerequisite to examining the incorporation and turnover of radioactive tracers in membranes was the development of procedures for the isolation of various membrane systems and membrane-bound organelles from the same population of cells. The characterization of several subcellular fractions by chemical, enzymatic and electron microscopic procedures will be presented. Utilizing these fractions, data will be put forth concerning the role of lipid precursors in membrane biosynthesis.

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MATERIALS AND METHODS

ORGANISMS: These experiments utilized the soil amoeba Acanthamoeba palestinensis maintained in Dr. R. N. Band's laboratory for several years.

Culture Medium: Cells were cultured under axenic conditions in Band's Amoeba Medium (1959). The medium contained 3 mg $MgCl_2.6H_2O$, 3 mg $CaCl_2$, 3 mg $FeSO_4.7H_2O$, 120 mg NaCl, 136 mg KH_2PO_4, 142 mg Na_2HPO_4, 18 g glucose and 15 g Proteose Peptone (Difco) to 1 L H_2O at a final pH of 6.8. The complete medium was sterilized by autoclaving for 20 min.

Culture Conditions: Cells were routinely cultured in 1 L silicone-coated Erlenmyer flasks containing 500 ml of medium. A constant temperature, rotaty shaker was used at 100 rpm and 29°C (Band and Machemer, 1963). Under the conditions described, cells grew with a dry weight doubling time of 27 hr. Only exponentially growing cells were utilized in experiments.

Gravimetric Measurements: Cell mass increase was determined by washing cells from 1 ml of medium once in cold distilled H_2O followed by desiccation over P_2O_5 and under vacuum in tared vials. The brief H_2O wash produced no cytolysis.

CELL FRACTIONATION: Aliquots of cells (3 to 5 ml wet, packed volume) were harvested, homogenized and fractionated according to a standardized scheme given below (Figure 1), in which TKM stands for 0.005M Tris, 0.025M KCl and 0.002M $MgSO_4$; and TM is a similar solution minus

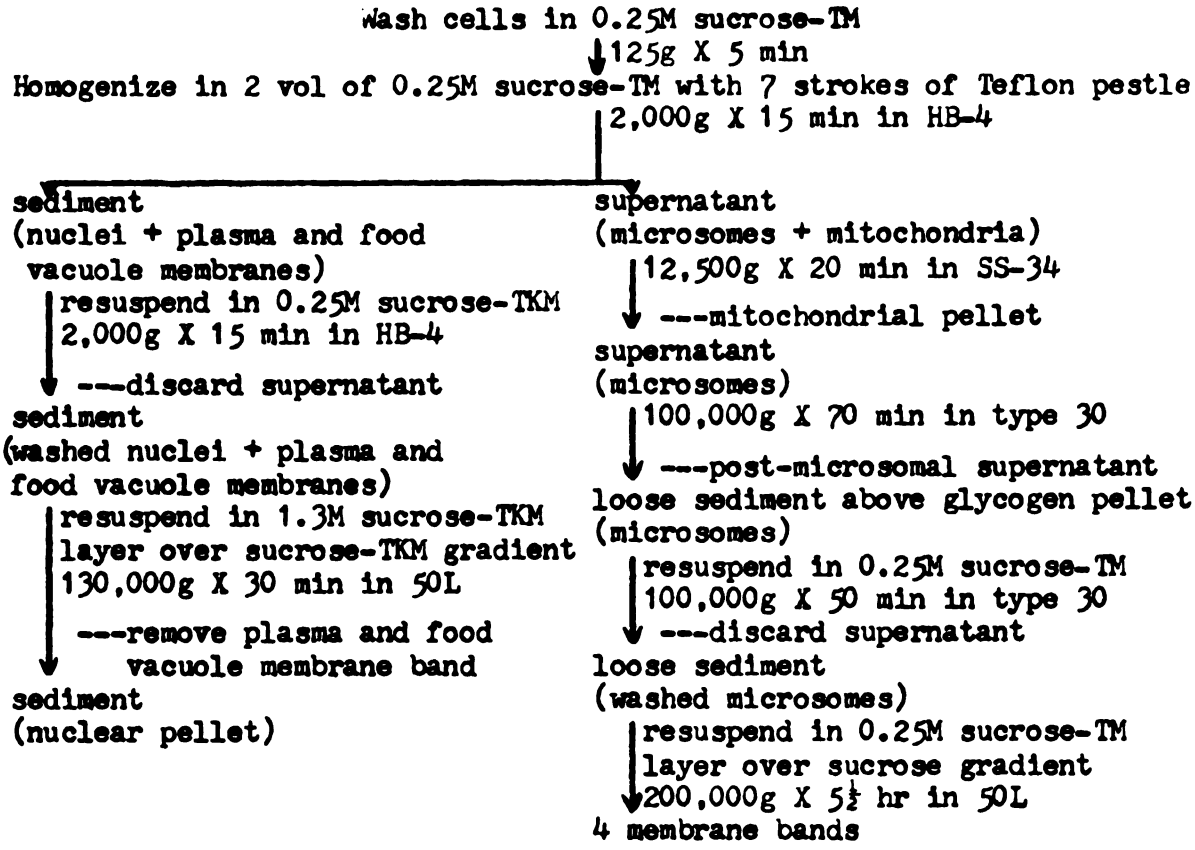

Figure 1. Standardized cell fractionation scheme. Values are average forces at the middle of the tube.

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KCl (cf. Blobel and Potter, 1966). All steps were carried out at pH 7.5, below 4°C.

Washing: Cells were washed once in 0.25M sucrose-TM and resuspended in the same solution to yield a final cell concentration of 1:2 (wet, packed volume:solution volume).

Homogenization: The cell suspension was homogenized with 7 strokes in a 30 ml capacity Potter-Elvehjem type Teflon grinder (A. H. Thomas Co.) with a clearance of 0.125-0.175 mm, driven by motor at 2,500 rpm.

Differential Centrifugation: For relative centrifugal forces less than 20,000g, a Sorvall RC-2 refrigerated centrifuge was used with either an HB-4 swinging bucket rotor or an SS-34 fixed-angle rotor. At forces exceeding 20,000g, a Spinco L2-50 ultracentifuge was used with either a 50L swinging bucket rotor or a type 30 fixed-angle rotor.

(a) The initial nuclear and plasma and food (digestive) vacuole membrane fraction was isolated from the 0.25M sucrose-TM homogenate and quickly transferred to, and washed once in, 0.25M sucrose-TKM to remove residual mitochondria.

(b) The mitochondrial fraction was immediately isolated from the post-nuclear supernatant, resuspended and washed 3 times in 0.25M sucrose-0.001M ethylenediamine-tetra-acetic acid (EDTA). According to Band and Morhlok (1969), the latter washings remove lysosomes and peroxisomes, as judged by the loss of acid hydrolase and catalase activity.

(c) Microsomes were separated from the post-mitochondrial supernatant by centrifugation at 100,000g X 70 min. The resulting microsomal fraction was very loosely layered above a large, almost transparent, glycogen pellet. The microsomes were removed from under the

post-microsomal supernatant witha pipette, gently resuspended in 0.25M sucrose-TM, and washed at 100,000g X 50 min. At the end of the run, the loosely layered microsomes were once again removed with a pipette.

(d) The post-microsomal supernatant with its floating non-membrane lipid layer (Stein and Shapiro, 1959) was recentrifuged at 100,000g X 50 min to remove any residual membranes.

Gradient Centrifugation: All linear sucrose density gradient centrifugations were performed in the Spinco L2-50 using a 50L swinging bucket rotor.

(a) The washed nuclear and plasma and food vacuole membrane pellet was gently resuspended by pipetting in 1 ml of 1.3M sucrose-TKM and layered over a 4 ml continuous sucrose-TKM gradient extending from 1.3M to 2.0M (1.17-1.27 g/cc at 4°C). The preparations were centrifuged at 130,000g X 30 min (cf. Blobel and Potter, 1966). At the end of this brief run, the microsomal contaminants were floating at the top of the tube, the plasma and food vacuole membranes formed a band in the gradient and the nuclei were pelleted at the bottom of the tube. The plasma and food vacuole membrane band, which extended from 1.18 to 1.195 g/cc was removed with a J-shaped needle attached to a syringe (Jamieson and Palade, 1967a). The residual supernatant, including the floating microsomal layer, was poured off, leaving the translucent pellet of pure nuclei. The plasma and food vacuole membrane preparation was diluted to 0.25M sucrose-0.001M EDTA, homogenized with 3 strokes of a 10 ml capacity Potter-Elvehjem type Teflon grinder (A. H. Thomas Co.) with a clearance of 0.10-0.15 mm at 1,000 rpm. This homogenate was washed twice in 0.25M sucrose-0.001M EDTA at 1,100g

X 10 min to yield the final plasma and food vacuole membrane pellet.

(b) Subfractionation of microsomes recovered from the differential centrifugations was accomplished by layering the microsomes, in 0.25 ml of 0.25M sucrose-TM (5 to 10 mg protein), over a 4.5 ml continuous sucrose density gradient (Britten and Roberts, 1960) extending from 1.04M to 2.0M (1.14-1.27 g/cc at 4°C), and centrifuging for 5½ hr at 200,000g (cf. Jamieson and Palade, 1967a). Upon termination of the run, the 4 distinct bands in the gradient (see Figure 7) were removed with a J-shaped needle attached to a syringe. The isolated fractions were either fixed for electron microscopy, washed for chemical analysis or precipitated with 10% trichloroacetic acid (TCA) prior to lipid extraction.

For cell free incorporation experiments, mitochondria, microsomes and the post-microsomal supernatant were isolated in the same manner, except that 0.25M sucrose-0.1M phosphate buffer (pH 7.4) was used in all steps.

RADIOACTIVE LABELING:

Choline-Methyl-C^{14}-Chloride (7.6 mc/m mole - New England Nuclear):

(a) Kinetics of Incorporation: In experiments invoving the kinetics of C^{14}-choline incorporation into TCA soluble and insoluble cell fractions, 10 ml of cells in culture medium (10^6 cells/ml) were incubated with 1 μc/ml (0.132 μ mole/ml). At appropriate time intervals, 1 ml aliquots of the medium were removed, diluted with ice-cold 0.25M sucrose-TM, and centrifuged to remove the cells. The cells were immediately resuspended and washed in the same solution. The washed cells were

brought to 1 ml (original volume) with cold 10% TCA and stored for a minimum of 2 hr below 4°C.

(b) Kinetics of Turnover: Turnover in TCA soluble and insoluble fractions was examined by "labeling" cells with 0.25 μc/ml (0.033 μ moles/ml) for 12 hr. The cells were then harvested, washed and resuspended in Amoeba Medium containing non-radioactive choline. The quantity of non-radioactive choline used was either 0.033 μ moles/ml (equal chase), 3.3 μ moles/ml (100X chase) or 16.5 μ moles/ml (500X chase). At appropriate time intervals, 1 ml aliquots of cells were removed and processed as described above.

(c) Cell Free Incorporation: Mitochondria, microsomes and post-microsomal supernatant, isolated in 0.25M sucrose-0.1M phosphate buffer, were brought to 1 mg protein/ml in 0.1M phosphate buffer (pH 7.4) with 1 μ mole $CaCl_2$/ml. Either 2 or 4 mg of protein from these suspensions was brought to 5.25 ml with a final mixture containing 5 μ moles $CaCl_2$ and 0.1M phosphate buffer. At time 0, 0.25 ml of phosphate buffer containing 1 uc (0.132 μ mole) of C^{14}-choline was introduced into the reaction mixture. After incubation at 29°C in a water bath with agitation, the reactions were terminated at specified time intervals by adding an equal volume of ice-cold 20% TCA (Vandor and Richardson, 1968). The 10% TCA solutions were stored at 2°C prior to lipid extraction. Methods for lipid extraction will be given in a subsequent section.

(d) Cell Free Turnover: The same methods described above were used, except that at certain times, concentrated, non-radioactive choline was introduced into the reaction mixture in 0.1 ml of phosphate buffer. As a control, 0.1 ml of phosphate buffer devoid of choline

was added simultaneously to identical mixtures. The concentration of non-radioactive choline was 66 μ moles (500X chase).

(e) Comparison of cell radioactivity to medium radioactivity: In experiments to determine the loss of radioactivity from cells into the medium, cells were labeled, washed and resuspended in medium containing non-radioactive choline as dexcribed for the kinetic studies of turnover. At sampling intervals, the total radioactivity of washed cells from 1 ml of medium (cell radioactivity) was determined by resuspending the cells to 1 ml (original volume) with distilled water and counting aliquots directly in scintillation fluid. In addition, the total radioactivity of 1 ml of medium, from which cells had been removed by centrifugation (medium radioactivity), was ascertained by directly counting aliquots of the medium in scintillation fluid.

(f) Turnover of C^{14}-Choline in Phospholipid of Cell Fractions: Cells were labeled as described above. In time sequence, aliquots of cells were fractionated into mitochondria, microsomes and post-microsomal supernatant. The fractions were precipitated with 10% TCA, washed and lipid extracted.

Glycerol-2-H^3 (500 mc/m mole - New England Nuclear):

(a) Kinetics of Incorporation: H^3-glycerol incorporation into TCA soluble and insoluble fractions was examined by labeling and processing cells in a manner identical to the C^{14}-choline studies. The only difference was that 5 μc/ml (0.01 μ mole/ml) of H^3-glycerol was used.

(b) Kinetics of Turnover: The kinetics of H^3-glycerol turnover in TCA soluble and insoluble fractions was monitored in a manner identical to the comparable studies with C^{14}-choline, except that 2.5 μc/ml (0.005 μ mole/ml) was used to label the cells. The non-radioactive

glycerol concentrations were 0.033 u mole/ml (6.6X chase), or 3.3 μ mole/ml (660X chase).

(c) Cell Free Incorporation: The conditions for cell free incorporation utilizing H^3-glycerol were as previously described for C^{14}-choline. 1.25 μc (0.0025 μ mole) of H^3-glycerol was present in the reaction mixture.

(d) Incorporation into Phospholipids of Cell Fractions: For these experiments, cells were concentated in 20 ml of Amoeba Medium at a ratio of 1:3 (cells:medium). Concentrations of 20 μc/ml (0.04 μ mole/ml), and in some cases 40 μc/ml (0.08 μ mole/ml), were added at time 0. In time sequence, aliquots of cells were fractionated according to the scheme in Figure 1. The cell fractions were precipitated with cold 10% TCA prior to lipid extraction.

(e) Turnover of Phospholipid in Cell Fractions: The experiments involving turnover of H^3-glycerol in phospholipid of cell fractions were carried out in a manner identical to similar studies with C^{14}-choline. Cells were labeled for 12 hr with 2 μc/ml (0.004 μ mole/ml) of H^3-glycerol, washed and resuspended in medium containing 3.3 μ mole/ml (825X chase) of unlabeled glycerol.

(f) Pulse and Chase of Phospholipid in Cell Fraction: Concentrated cell suspensions were suspended in medium containing 30 μc/ml (0.06 μ mole/ml) for 10 min. Then, the cells were removed from the medium by centrifugation, washed once in Amoeba Medium, and finally, resuspended in medium with 16.5 μ mole/ml (275X chase) of non-radioactive glycerol. At suitable time intervals, aliquots of cells were fractionated as described.

RADIOACTIVE ASSAYS:

Liquid Scintillation: All radioactive counting was done in a scintillation fluid containing 7.84 g 2,5-diphenyloxazole (PPO), 0.16 g p-bis-(o-methylstyryl)-benzene (bis-MSB) and 120 g naphthalene to 1 L of p-dioxane. All counting rates were corrected for background and quenching to disintegrations per minute (dpm). Counting was done on a Beckman Mark III refrigerated liquid scintillation counter.

CO_2 Counting: Gases derived from the radioactive medium in which cells were growing was passed through a 0.1N NaOH trap over a 3 day period. At the end of this time interval, aliquots of the NaOH solution with trapped CO_2 were counted directly in the scintillation solution.

Radioactivity of Lipid Extracts: Aliquots of radioactive lipid extracts in chloroform:methanol (2:1) were placed in scintillation vials and allowed to evaporate to dryness. The scintillation fluid was added and the vials counted as described.

Radioactivity of TCA Soluble and Insoluble Fractions: The washed cells from 1 ml of medium, which had been brought to exactly 1 ml with 10% TCA, were left in this suspension for at least 2 hr at 2°C (see sections on kinetic studies). The TCA insoluble fraction was centrifuged down at 16,000g X 5 min. Aliquots of the clarified cold TCA soluble fraction were removed and counted directly in the dioxane-base fluid. The TCA precipitate of the cells from 1 ml of medium was washed twice in cold 5% TCA and once in cold H_2O to remove any residual insoluble radioactivity. The TCA insoluble material was dissolved in 88% formic acid and transferred to counting vials. Thus, values can be related to 1 ml of medium and are expressed as dpm/cells/ml of medium.

LIPID EXTRACTION AND CHROMATOGRAPHY:

Lipid Extraction: To insure complete removal of insoluble radioactivity, lipids were extracted from washed TCA precipitates (Dallner, Siekevitz and Palade, 1966a). The original 10% TCA insoluble precipitates were washed by centrifugation twice with cold 5% TCA and once with cold distilled water. The precipitates were then lipid extracted at room temperature for 2 hr in chloroform:methanol (2:1). The extracts were separated from the insoluble residue and washed with 0.1N HCl (5 ml/4 ml extract) to remove any protein remaining. Separate aliquots were used for lipid phosphorus determinations, scintillation counting or chromatographic analysis.

Chromatography of Phospholipids: Lipids in the chloroform:methanol extract were spotted on activated silica-gel G plates and developed in chloroform:methanol:acetic acid:water (25:15:4:2) for 90 min (Randerath, 1966). The separated phospholipids were detected by iodine vapors and scraped into scintillation vials for counting.

CHEMICAL ANALYSIS:

Protein: Using bovine serum albumin as a standard, protein was determined by the Folin-Ciocalteau test (1927).

Ribonucleic Acid: RNA was determined on washed TCA precipitates with the Mejbaum (1939) orcinol technique, utilizing hot (90°C) TCA extraction of nucleic acids as proposed by Schneider (1946). Purified yeast RNA was used as a standard.

Lipid Phosphorus: Lipid phosphorus was measured according to Marinetti (1962) using 70% perchlorate at 150°C to digest the lipid

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Inorganic Phosphorus: Monophosphates liberated in the phosphatase assays were measured by the Fiske and Subbarow method (see Lindberg and Ernster, 1956).

ENZYME ANALYSIS: Allenzymes examined were phosphatases and were assayed by the liberation of inorganic phosphates. Values are expressed as μ moles Pi/mg protein/hr.

Glucose-6-Phosphatase: This enzyme was assayed by a modification of the method of Swanson (1955). The reaction mixture contained 250 ug cell fraction protein, 2×10^{-4} moles disodium glucose-6-phosphate (Sigma Chemical Co.), 1×10^{-5} moles histidine and 1×10^{-6} moles EDTA in 1 ml of H_2O at pH 6.5. After incubation at 29°C for 30 min, the reaction was terminated by the addition of 0.5 ml of cold 20% TCA with rapid cooling of the tubes. The TCA precipitate was centrifuged down and the liberated inorganic phosphorus in the supernatant determined. Blanks were processed similarly, except that no substrate was present.

Thiamine Pyrophosphatase: The method of Novikoff and Heus (1963) was used to determine this enzyme's activity. The assay mixture contained 250 ug cell fraction protein, 6×10^{-7} moles of thiamine pyrophosphate chloride (Sigma Chemical Co.), 2.4×10^{-5} moles of Tris-HCl and 1.25×10^{-6} moles of $MgCl_2 \cdot 6H_2O$ in a volume of 1 ml at pH 7.4. The other aspects of the assay were identical to those described for glucose-6-phosphatase.

Magnesium-Dependent Adenosine Triphosphatase: Wallach and Ullrey's (1964) method was used to analyze this enzyme as well as sodium and potassium-activated, magnesium-dependent adenosine triphosphatase. The reaction mixture contained 250 ug cell fraction protein, 5×10^{-7} moles Tris-adenosine triphosphatase (Sigma Chemical Co.), 1×10^{-5} moles Tris-HCl, 5×10^{-8} moles EDTA and 5.5×10^{-6} moles $MgCl_2 \cdot 6H_2O$ in a volume of 1 ml at pH 8.4. The other parts of the assay procedure were identical to the other phosphatase methods described.

Sodium and Potassium-Activated, Magnesium-Dependent Adenosine Triphosphatase: The reaction mixture was the same as for magnesium-dependent adenosine triphosphatase, except that it contained 1.02×10^{-5} moles NaCl and 5×10^{-6} moles KCl. The analysis procedures were the same.

MICROSCOPY: Pellets of cell fractions were fixed *in situ* in 5% glutaraldehyde with 0.1M phosphate buffer (pH 7.2) at $4^{\circ}C$ for 12 hr. These samples were rinsed in cold phosphate buffer and post-fixed for 45 min at $4^{\circ}C$ in Zetterqvist's osmium fixative (see Pease, 1964). Pellets of cells were fixed in osmium without pre-fixation in glutaraldehyde. All specimens were dehydrated in ethanol and embedded in Araldite. Sections were cut with glass knives on a Porter-Blum MT-2 microtome, mounted on carbon coated or uncoated grids and stained with uranyl acetate, followed by lead citrate. Sections were examined and micrographed in a Hitachi HU-11E operated at 75kv with a double condenser. Preparations were scanned and micrographed at direct magnifications ranging from 10,000 to 40,000X.

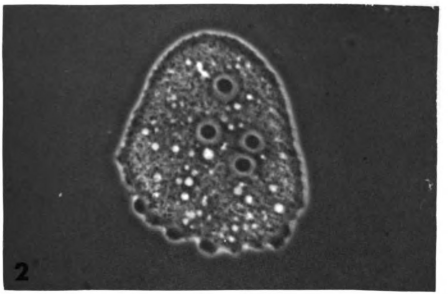
RESULTS

DESCRIPTION OF *ACANTHAMOEBA PALESTINENSIS*:

Morphology: Populations of cells grown in a state of continuous suspension in Amoeba Medium are heterogeneous with respect to cell size, nuclear size and the presence or lack of multinuclearity (Band and Machemer, 1963). As can be seen in Figures 2 and 3, nuclei with associated nucleoli, numerous vacuoles and small particles (mostly mitochondria) are visible utilizing phase contrast microscopy. Ultrastructurally (Figures 4 and 5), *Acanthamoeba* appears to contain a typical array of cytoplasmic organelles (see also Bowers and Korn, 1968, for a complete analysis of the fine structure of *Acanthamoeba*). Fine structural observations reveal no extracellular coat like those of *Amoeba proteus* and *Chaos chaos*. The ultrastructurally distinct membranes are: (1) the inner and outer portions of the nuclear envelope, (2) the inner and outer mitochondrial membranes, (3) the large vacuole membranes (e.g. food (digestive) and contractile vacuole membranes), (4) the small vesicle membranes (e.g. pinocytotic vacuoles, lysosomes and peroxisomes), (5) the Golgi complex membranes, (6) the smooth endoplasmic reticulum (primarily in the form of elongate cisternal elements, as well as tubular membranes), (7) the rough endoplasmic reticulum and (8) the cell surface membrane. Osmiophilic lipid droplets, which probably represent most of the non-membrane lipids, are evident in these cells. Copious amounts of glycogen particles are also visible in the cytoplasmic matrix.

Figure 2. Phase contrast micrograph of multinucleate Acanthamoeba palestinensis. X 6,000.

Figure 3. Phase contrast micrograph of a group of amoeba cells. Note the binucleate cell undergoing synchronous nuclear mitosis. X 6,500.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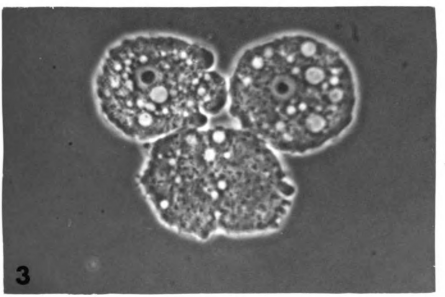
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Figure 4. Electron micrograph of portion of Acanthamoeba cell illustrating nuclear envelope (N), mitochondria (M), digestive vacuole (D), small, cisternal smooth membranes (C) and cell surface membrane (CM). X 32,000.

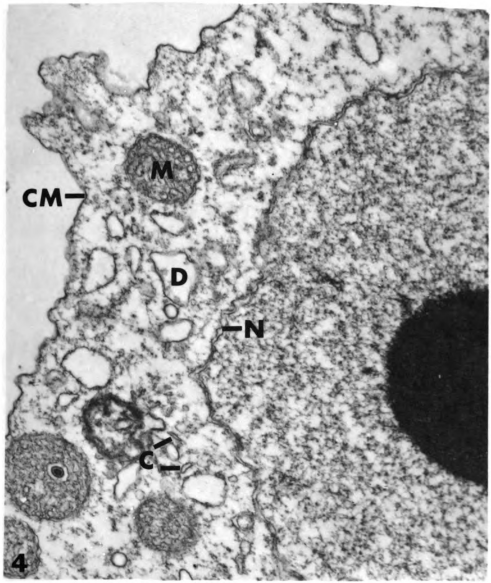
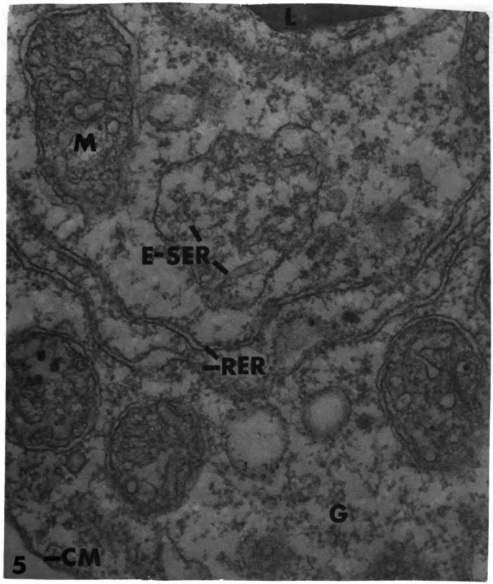

M
CM
D
N
C
4

Figure 5. Electron micrograph of portion of Acanthamoeba cell. Note the edge of a lipid droplet (L), glycogen particles (G), rough endoplasmic reticulum (RER), profiles and cross sections of elongate, tubular smooth endoplasmic reticulum (E-SER), mitochondria (M) and cell surface membrane (CM). X 68,000.


L
M
E-SER
RER
G
5 CM

Growth: Cells grown in silicone-coated shaker flasks exhibited a 27 hr generation time (Figure 6). Due to the variations in cell size, cell number counts were not as precise as dry weight determinations in checking growth rates. The average number of cells per mg dry weight was $0.64 \pm 0.05 \times 10^6$. Cells inoculated into Amoeba Medium began exponential growth without any noticeable lag phase.

CELL FRACTIONATION:

Explanation of Techniques: The primary criteria employed in developing the fractionation procedure (Figure 1) were the preservation and purity of the isolated membrane systems and membrane bound organelles. Since all of the work with these fractions will be presented in terms of specific activities, total recovery was not considered to be an important factor.

Homogenization was a critical step in the fractionation scheme. The procedure used produced the breakage of a maximum number of cells, while preserving the released cellular organelles and membrane systems. The homogenization medium of 0.25M sucrose-TM was used as a compromise to allow all fractions to be isolated from the same homogenate. It was found that nuclei were most easily isolated from cells homogenized in 0.25M sucrose-TKM (cf. Hlobel and Potter, 1966); but the K^+ ions destroyed the mitochondria and affected the densities of the microsomes (cf. Dallner and Nillson, 1966). Mitochondria, on the other hand, could have been isolated intact, from the post-nuclear supernatant of cells homogenized in 0.25M sucrose-0.001M EDTA. However, the lack of divalent catios and proper ionic strenght would have caused

Figure 6.

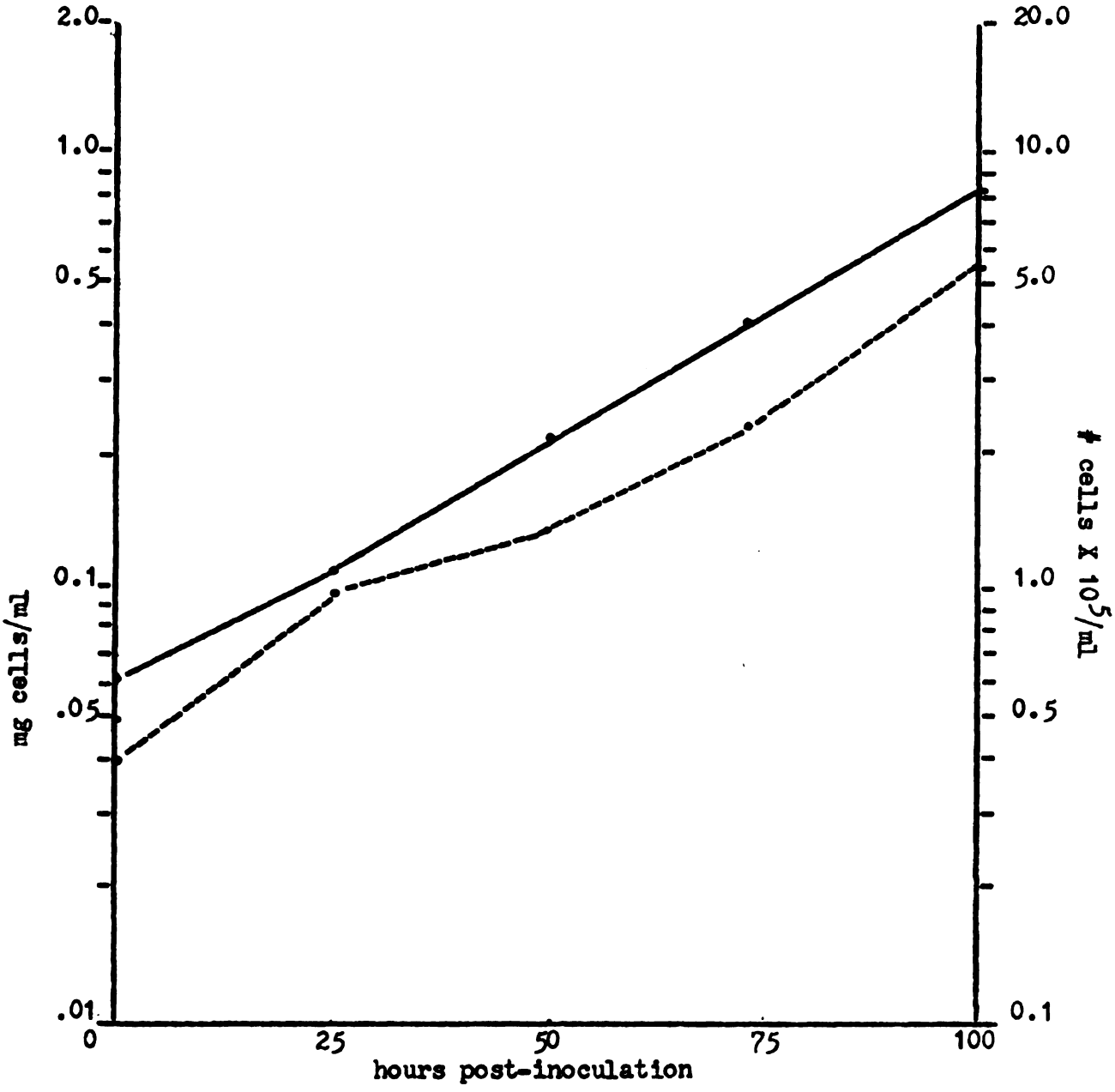


Figure 6. Growth of Acanthamoeba palestinensis in shaker flasks. The broken line (---)represents # cells/ml and the unbroken line (___) represents mg cells/ml.

the nuclei to aggregate, swell and break (cf. Umbreit, Burris and Stauffer, 1957). The compromise homogenization medium settled upon (0.25M sucrose-TM) was isotonic enough to allow the nuclei to remain in it briefly, and contained a small amount of divalent cations, which prevented the nuclei from clumping. The presence of divalent cations did cause some adherence of mitochondria to membrane fragments; but this process was reversed by the eventual washings of the mitochondria in 0.25M sucrose-0.001M EDTA. The washings caused many mitochondria to swell and lyse.

Immediately following homogenization, the nuclei, along with the plasma and food vacuole membranes, were separated from the homogenization medium by centrifugation. The light colored top layer of the resulting pellet, containing most of the nuclei and plasma and food vacuole membranes, was quickly resuspended and washed in 0.25M sucrose-TKM (Figure 7). The bottom of the initial pellet, containing unbroken cells, was discarded. In isolation medium containing TKM, the nuclei were quite stable. However, this medium caused the plasma and food vacuole membranes to shrink greatly. After the centrifugal washing, the nuclear and plasma and food vacuole membrane pellet was resuspended in 1.3M sucrose-TKM and subjected to gradient centrifugation as described in the methods. The passage of nuclei down the gradient was sufficient to rip off and float up any cytoplasmic rough endoplasmic reticulum attached to the nuclei. Hence, a pure, translucent nuclear pellet was obtained (Figure 8). The linear gradient was steep enough to allow the plasma and digestive vacuole membranes to band above the nuclear pellet at 1.18 to 1.19 g/cc at 4^{o}C (Figure 7). This band was

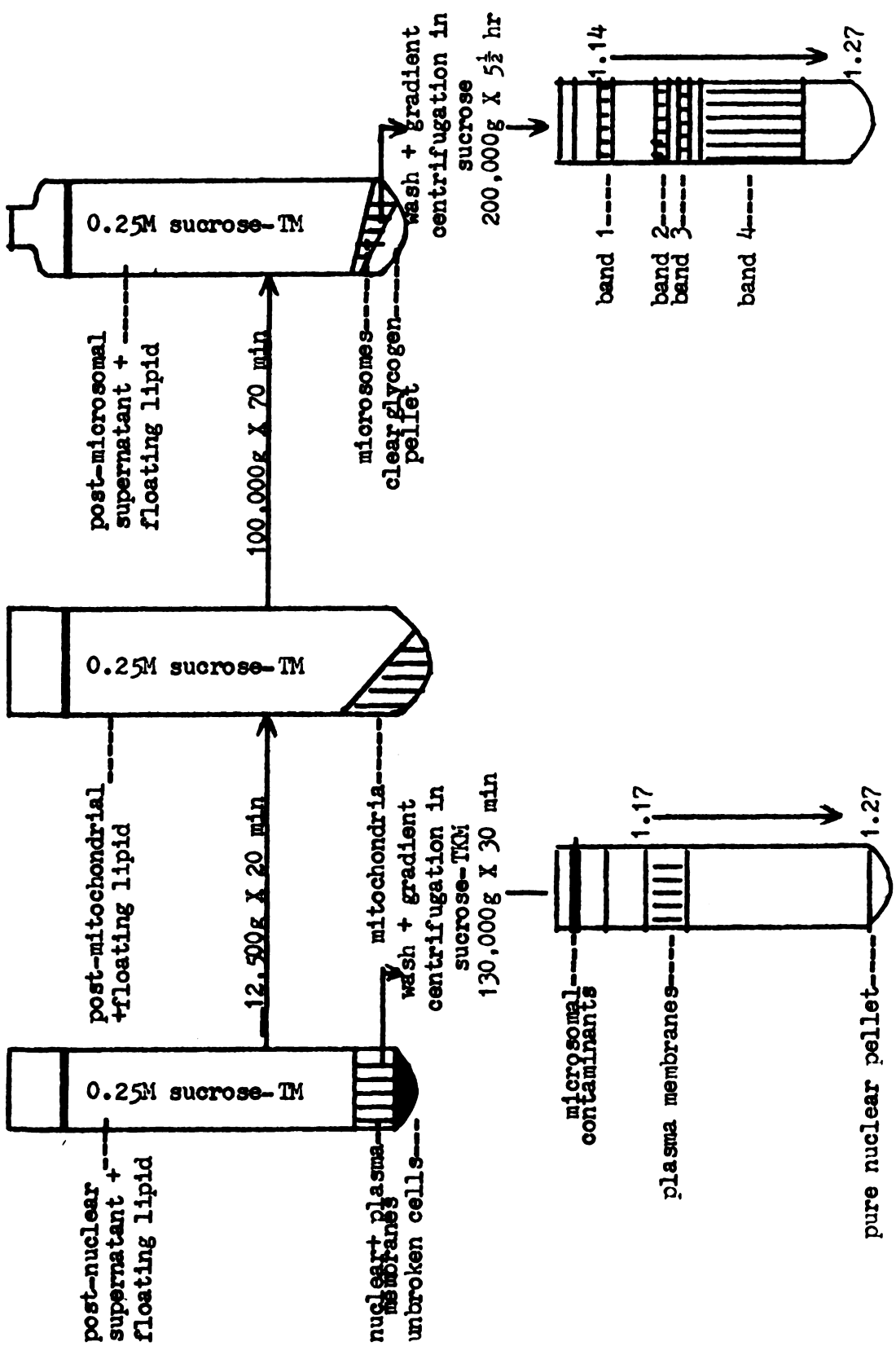
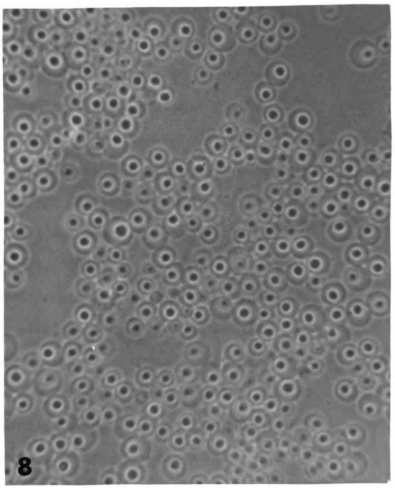


Figure 7. Diagramatic representation of some centrifugal steps in the fractionation procedure.

Figure 8. Phase contrast micrograph of nuclear fraction. X 4,500.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removed, rehomogenized and washed in 0.25M sucrose-0.001M EDTA. This washing, in addition to removing mitochondrial and microsomal contaminants, allowed the membranes to return to a sac-like shape and caused vesiculation.

Mitochondria were separated from the post-nuclear supernatant by high speed centrifugation (Figure 7) which, while insuring the removal of the mitochondria from the post-mitochondrial supernatant, also increased the sedimentation of contaminating microsomes. These contaminants were removed by the subsequent, three low speed washings at 10,000g in 0.25M sucrose-0.001M EDTA. In addition, the washings of the mitochondrial fraction removed peroxisomes and lysosomes (Band and Morhlok, 1969), which are common contaminants of mitochondrial preparations (deDuve, 1967).

The duration of the differential centrifugations involving separation of the microsomes from the post-mitochondrial supernatant, and their subsequent washing, was a critical factor in the preservation of the membrane systems. It was essential that the lenght of these 100,000g centrifugations be adjusted such that the microsomes were gently layered over the glycogen pellet at the end of each run (Figure 7). If the microsomes were packed tightly over the glycogen pellet at the end of each differential centrifugation, not only were microsomes lost into the glycogen pellet; but distinct banding patterns were not obtained in the subsequent gradient centrifugation. It was observed that the presence or lack of Mg^{++} ions in the gradient had no effect on equilibrium densities. Figure 7 illustrates the banding pattern routinely obtained using the described methods.

Morphology of Fractions: When viewed with a phase microscope (Figure 8) the nuclear fraction appeared homogeneous. Electron micrographs (Figure 9) demonstrated that no cytoplasmic rough endoplasmic reticulum remained adhering to the outer rough nuclear membrane. In most cased the inner and outer nuclear membranes were left intact. Some nuclei were observed with portions of the outer rough nuclear membrane sheared off.

Phase contrast scanning of the plasma and digestive vacuole membrane fraction in 0.25M sucrose-TKM revealed a highly shrivelled state induced by the medium. At this time it is uncertain whether this was a contractile phenomena or an osmotic shrinking. However, the same event was encountered when cells were homogenized directly in 0.25M sucrose-TKM. In the latter case the ionic strength inside and outside the membrane vesicles would be expected to be the same. If these same membranes were washed several times in 0.25M sucrose-0.001M EDTA, they regained a sac-like shape and vesiculated. It is interesting that the equilibrium density of the membranes was unaltered by the presence or lack of the K^+ ions. Ultrastructurally, plasma membranes could not be distinguished from the food vacuole membranes (Figure 10). Only trilamellar structures of the membrane vesicles were observed when the membranes were cut at normal angles.

The mitochondrial pellet recovered after 3 washings in 0.25M sucrose-0.001M EDTA contained intact and swollen mitochondria (Figure 11). Intact mitochondria exhibited a highly characteristic structural organization. The matrix of the mitochondria stained intensely. Vesicles of swollen mitochondria usually retained this matrix, indicating

Figure 9. Electron micrograph of the nuclear fraction. X 24,000.

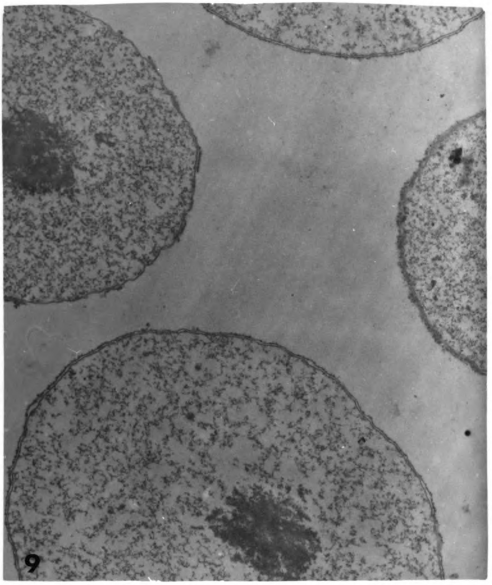
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Figure 10. Electron micrograph of the plasma and digestive vacuole membrane fraction. X 67,500.

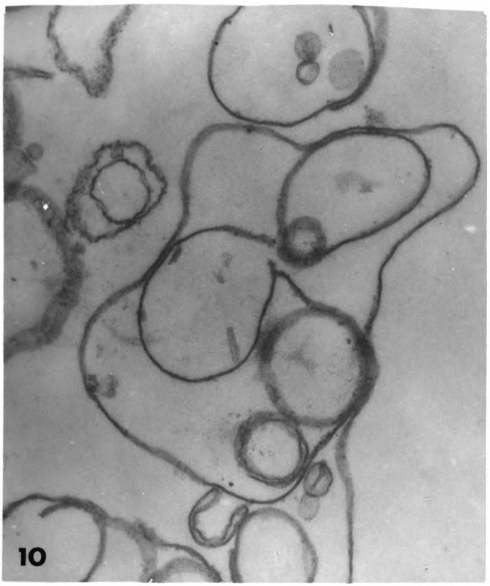
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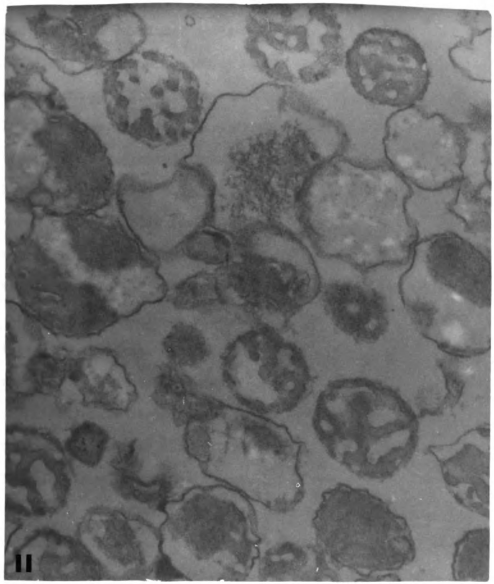
Figure 11. Electron micrograph of the mitochondria fraction. X 60,000.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their origin.

Band 1, recovered from the continuous gradient centrifugation of the microsomes (see Figure 7), was the least dense (1.14-1.15 g/cc at 4°C) and was composed almost entirely of smooth surfaced elements (Figure 12). These smooth membranes appeared to be in a multitude of configurations, ranging from large vesicles, usually containing a smaller vesicle, to curved, cisternal structures and long, narrow, tubular structures. It is suggested that the images of a vesicle within a vesicle are, in reality, tangential cuts across one of the two primary structures composing this fraction. This structure can be likened to a thin-surfaced, hollow, sphere, which had been folded in upon itself. The curved, cisternal structures are, in fact, different profiles of these same structures. These elements fit the current cytological description of the intact, elongate, smooth endoplasmic reticulum. Membranes are also present in narrow tubular configurations. Bowers and Korn (1968) have described this type of smooth endoplasmic reticulum in Acanthamoeba (see also Figure 5).

The majority of membranes in band 2 (1.175 g/cc at 4°C) were in a small cisternal configuration (Figure 13). However, these smooth membrane elements, unlike those of band 1, were only about 1,500 Å in length. Small particles were also present. The chemical analysis (see Table 1) suggests that these particles are most likely ribonucleoprotein. The bottom-most edge of the pellet of this band (Figure 14), in addition to containing the small, cisternal elements, also contained larger vesicles in distended shapes. It is suggested that these membranes might be damaged Golgi membrane contaminants from the top of band 3.

Figure 12. Electron micrograph of the elongate type of smooth endoplasmic reticulum from pellet of band 1. X 60,000.

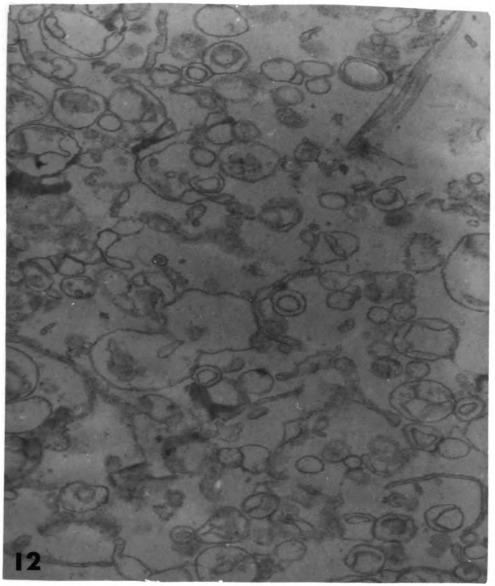
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Figure 13. Electron micrograph of the small, cisternal smooth membranes. Section was from the middle of band 2. X 110,000.

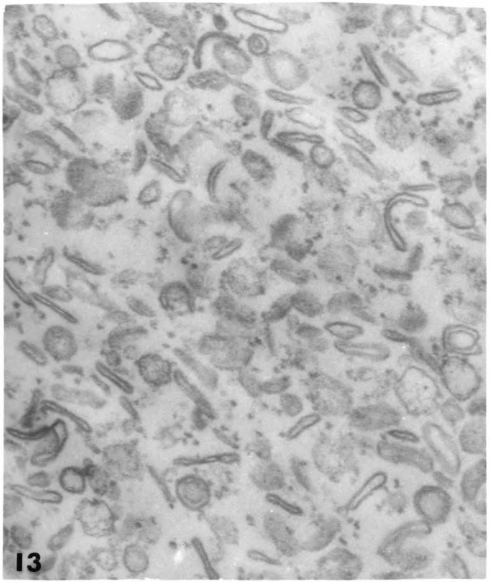
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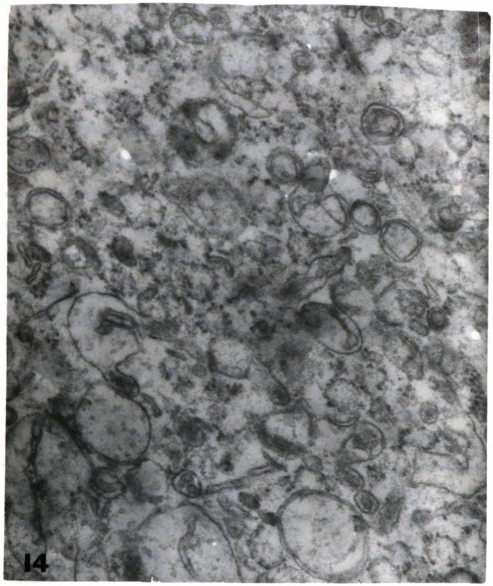
Figure 14. Electron micrograph of the Golgi membrane contaminants at the bottom of the pellet of band 2. X 60,000.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Band 3 (1.183 g/cc at 4°C) was composed primarily of Golgi complex membranes sectioned at various angles. Some vesicular rough endoplasmic reticulum (Figure 15) was also present. The distended ends of the Golgi profiles were often filled with an electron dense material. The amount of rough surfaced vesicles increased at the bottom of the pellet of this band (Figure 16).

Band 4 (1.194-1.208 g/cc at 4°C) was composed almost entirely of rough surfaced membranes (Figure 17). In some instances matrix material was seen. Very few free ribosomes were present. The rough endoplasmic reticulum was usually in the form of large, distended, shapes, rather than small, round vesicles.

Chemical Composition of Membranes:

(a) RNA/protein ratios: Table 1 gives the RNA/protein ratios of

Table 1. RNA/protein ratios of membrane fractions.*

Membrane Fraction**	Expt. 1	Expt. 2	Expt. 3	Average
E-SER	0.05	0.05	0.05	0.05
C-SM	0.09	0.09	0.11	0.10
G	0.10	0.10	0.19	0.13
RER	0.24	0.22	0.28	0.25
PDM	0	0	0	0

*values expressed in µg RNA/µg protein
**E-SER = elongate smooth endoplasmic reticulum from band 1, C-SM = small, cisternal smooth membranes from band 2, G = Golgi membrane fraction of band 3, RER = rough endoplasmic reticulum of band 4 and PDM = plasma and digestive vacuole membrane fraction

Figure 15. Electron micrograph of the Golgi membrane fraction. Section was from the middle of the pellet of band 3. X 60,000.

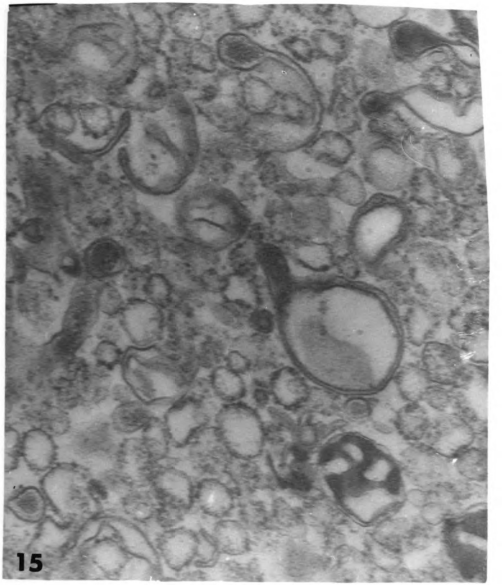
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Figure 16. Electron micrograph of the bottom of the pellet of band 3 demonstrating the rough endoplasmic reticulum contamination. X 67,500.

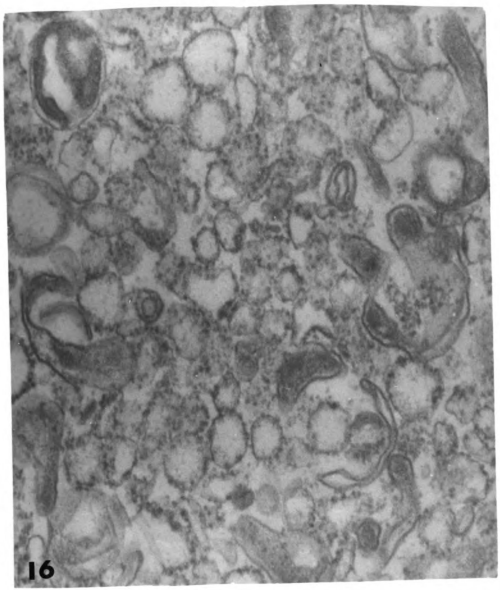
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Figure 16. Electron micrograph of the bottom of the pellet of band 3 demonstrating the rough endoplasmic reticulum contamination. X 67,500.

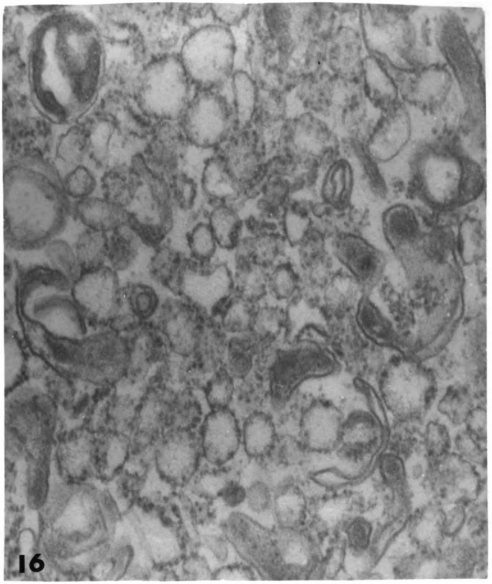

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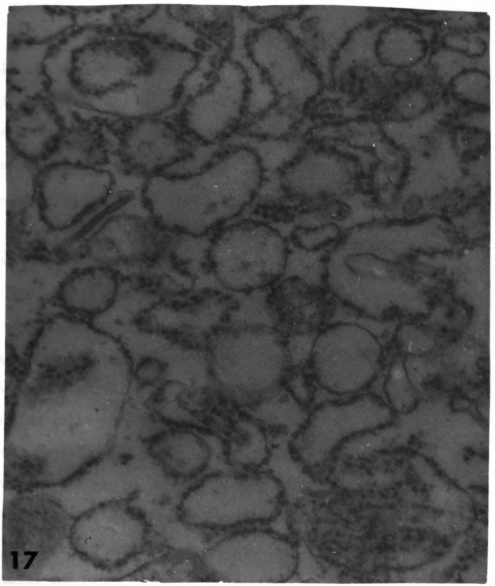
Figure 17. Electron micrograph of the rough endoplasmic reticulum composing band 4. X 82,500.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the membrane fractions isolated. The values of three separate determinations on the fractions isolated from 3 different cell populations are given.

(b) Phospholipid composition of cells and microsomes: Both whole cells and washed microsomes were lipid extracted and chromatographed as described in the methods section. The phospholipids found in each case were identical. Phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, and phosphatidyl inositol were identified (Figure 18). Triglycerides, fatty acids, phosphatidic acid and sterols would not be detectable by the chromatographic procedure used, and would all appear in the spot at the solvent front.

Preliminary Enzyme Characterization of the Cell Fractions: Since the enzymes of Acanthamoeba have not been extensively investigated, no a priori method was available to enzymatically classify the membrane fractions. Several enzymes commonly used as membrane markers in mammalian cells were employed. The specific activities of the enzymes in each fraction are given as u moles of inorganic phosphorus hydrolyzed per hour per mg protein (μ moles Pi/hr/mg protein) in Table 2. The results of 2 experiments are given.

The specific activity of glucose-6-phosphatase was enhanced in the Golgi and rough endoplasmic reticulum fractions, relative to the specific activity observed in the homogenate. The elongate type of smooth endoplasmic reticulum and the small, cisternal smooth membranes contained no activity. The highest specific activity (10X higher than the homogenate) was observed in the plasma and digestive vacuole membrane fraction prepared by the described methods. If the plasma

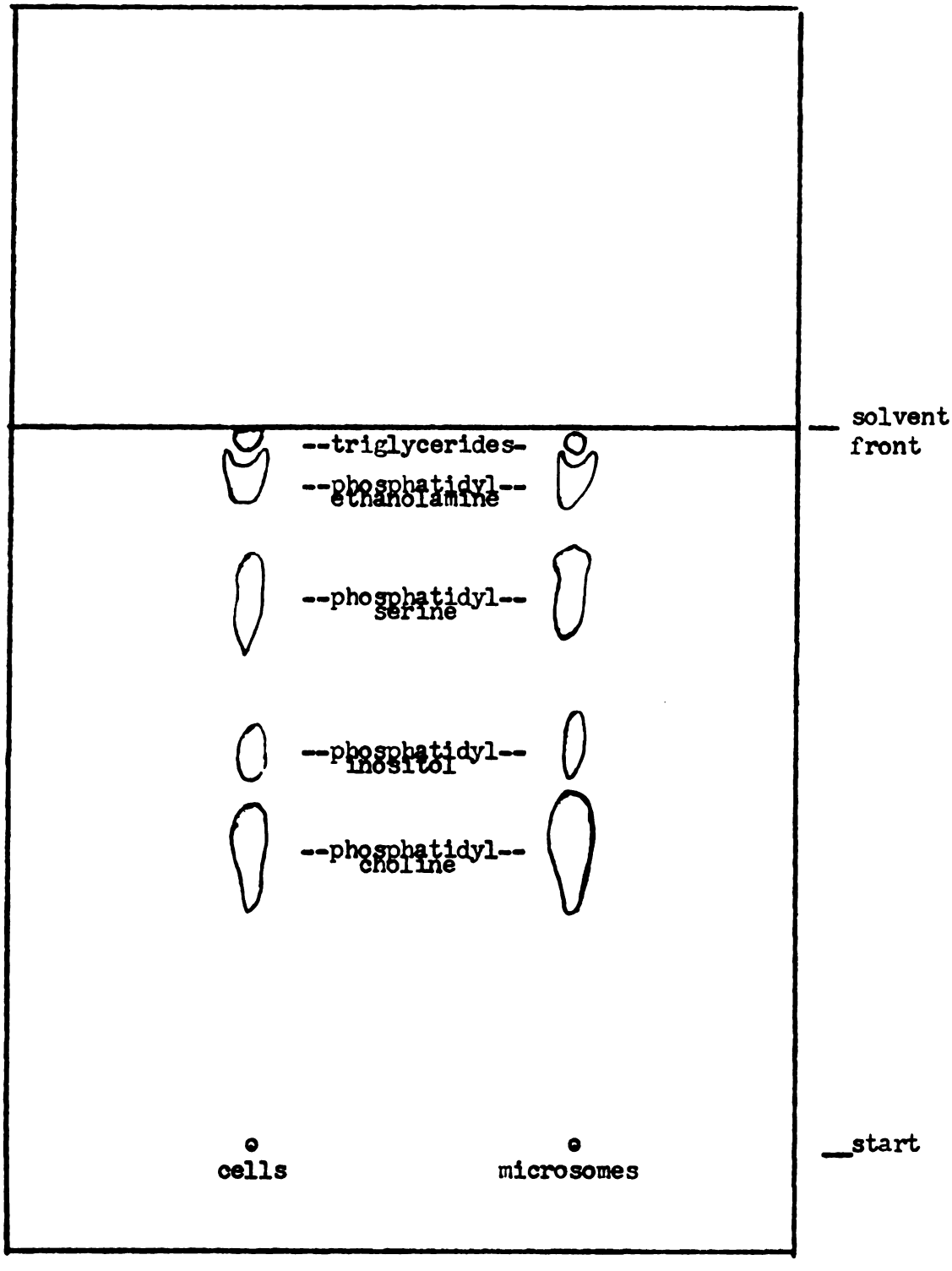

Figure 18. Chromatography of phospholipids on silica gel plate.

Table 2. Specific activities of enzymes in cell fractions.*

Fraction**	Expt. #	G-6-Pase	TPPase	Mg^{++}-ATPase	Na^{+}/K^{+}-ATPase
H	1	0.042	0.038	0.151	0.181
	2	0.049	0.036	0.109	0.123
N	1	0.028	0	0.233	0.217
	2	0.017	0	0.233	0.228
M	1	0.104	0.039	0.425	0.201
	2	0.090	0.032	0.476	0.210
E-SER	1	0	0.261	0.413	0.555
	2	0	0.109	-	-
C-SM	1	0	0.439	0.610	0.696
	2	0	0.417	0.566	0.613
G	1	0.177	0.446	0.572	0.686
	2	0.177	0.389	0.542	0.428
RER	1	0.168	0.143	0.271	0.259
	2	0.153	0.192	0.222	0.321
PDM (EDTA)	1	0.484	0.476	0.773	0.776
	2	0.480	0.476	0.873	0.933
PDM (TKM)	1	0.274	0.312	0.659	0.549
	2	0.297	0.140	0.742	0.710
PMS	1	0.105	0.049	0.144	0.100
	2	0.116	0.039	0.102	0.100

*values expressed in μg Pi/hr/mg protein

**H = homogenate, N = nuclear fraction, M = mitochondrial fraction, E-SER = elongate smooth endoplasmic reticulum, C-SM = small, cisternal membranes, G = Golgi fraction, RER = rough endoplasmic reticulum, PDM (EDTA) = plasma and digestive vacuole membrane fraction washed in 0.25M sucrose-0.001M EDTA, PDM (TKM) = plasma and digestive vacuole membrane fraction washed in 0.25M sucrose-TKM and PMS = post-microsomal supernatant

and digestive vacuole membranes had been washed in 0.25M sucrose-TKM, rather than 0.25M sucrose-0.001M EDTA, prior to the analysis, the specific activity was decreased by about 50%. A small enhancement of enzyme activity was found in the mitochondrial fraction. As can be seen from the specific activity in the post-microsomal supernatant, some of the enzyme was soluble.

While the elongate smooth and the rough endoplasmic reticulum exhibited some thiamine pyrophosphatase activity, the small, cisternal smooth membranes, Golgi membranes and plasma and digestive vacuole membranes were observed to contain the largest amount of this enzyme. Again, TKM washings of the plasma and digestive vacuole membrane fraction resulted in a decrease in activity. The nuclear and mitochondrial fractions demonstrated no activity. As can be judged by the small amount of activity in the post-microsomal supernatant, very little of the enzyme was soluble.

Magnesium-dependent adenosine triphosphatase activity was present in all the membrane containing fractions, indicating that practically all of the enzyme was particulate. The highest specific activities were found in the plasma and digestive vacuole membrane, Golgi membrane and small, cisternal membrane fractions. However, no increase in the latter values was produced by sodium and potassium ions in any of the fractions (compare Na^{+}/K^{+}-ATPase values to Mg^{++}-ATPase values in Table 2). A 50% decrease in activity was observed in the mitochondrial fraction, most likely due to potassium loading. Sodium and potassium stimulation of magnesium-dependent adenosine triphosphatase is often used as a criterion for the detection of plasma

membranes (Novikoff, Essner, Goldfischer and Heus, 1962). The results in Table 2 indicate that TKM washings had no deleterious effect upon adenosine triphosphatase activity.

EXPERIMENTS WITH C^{14}-CHOLINE

Specificity of Labeling:

(a) Measurement of the radioactivity of medium with growing cells: To detect whether any radioactivity was being lost from the growth medium to the atmosphere, 1 ml portions of radioactive medium were counted over an experimental period of 4 days. No changes in radioactivity were detected in 2 separate experiments. This indicated that no radioactivity was lost to the atmosphere from the culture of growing cells.

(b) CO_2 trapping: As a check on the above results, the CO_2 gas, from cells grown in 0.25 µc/ml of C^{14}-choline for 3 days, was trapped in 1N NaOH and counted. In 2 experiments, no radioactivity was detected. Thus, it is certain that none of the C^{14}-choline is metabolized down to the state of $C^{14}O_2$ under the growth conditions described.

(c) Recovery of radioactivity in lipid extracts: Three populations of cells grown in 0.25 µc/ml of C^{14}-choline for 3 days were washed and lipid extracted as described. Total radioactivity of the lipid extract and the lipid-extracted TCA precipitates was compared. The results in Table 3 illustrate that essentially all of the TCA insoluble radioactivity was lipid extractable.

Table 3. Recovery (%) of C^{14}-radioactivity in lipid extracts of cells.*

Expt. #	Lipid Extract of TCA Ppt.	Extracted TCA Ppt.	% Recovery
1	482,500	4,000	99.2%
2	212,100	597	99.7%
3	358,500	1,854	99.5%

*values given in dpm

(d) Chromatography of radioactive lipid extracts: The lipid extracts of whole cells, as well as microsomes isolated from cells, labeled for 1 day in 0.25 μc/ml of C^{14}-choline were chromatographed as described (see Figure 18). The radioactivity of the spots was determined in 2 separate experiments. The results, illustrated in Table 4, demonstrate that C^{14}-choline was a specific label for phosphatidyl choline.

Table 4. Localization of C^{14}-choline in phosphatidyl choline by chromatography.*

Sample	Expt. #	PC	PI	PS	PE	SF
Cells	1	1,282	0	0	0	0
	2	420	0	0	0	0
	1	19,000	0	0	0	39
	2	6,890	0	0	0	0

*values given in dpm; PC = phosphatidyl choline, PI = phosphatidyl inositol, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine and SF = spot at solvent front.

(e) Measurement of lipid radioactivity of medium: Radioactive medium (0.25 μc/ml of C^{14}-choline), in which cells had been grown for 3 days, was cleared of cells by low speed centrifugation. The lipids extracted from the TCA insoluble fraction of the medium were counted as described. No lipid radioactivity was found in two experiments.

It thus seems that all of the C^{14}-choline utilized by the cells was incorporated into phosphatidyl choline, was precipitatable with TCA and was extractable with lipid sovents. Therefore, direct counting of TCA precipitates was utilized to give an approximation of the C^{14}-choline present as phosphatidyl choline.

Characteristics of C^{14}-Choline Incorporation and Turnover:

(a) Kinetics of incorporation into TCA soluble and insoluble fractions: By utilizing washed cells derived from the same volume of medium (1 ml) and by determining the total radioactivity of both the TCA soluble and insoluble fractions of these cells, as described in the methods, no corrections for growth dilution of radioactivity were necessary. Figures 19 and 20 illustrate that incorporation into the TCA insoluble fractions of 2 different cell populations became linear about 45 min after introduction of 1 μc/ml of C^{14}-choline. Coinciding with the beginning of linear incorporation into the phosphatidyl choline of the TCA insoluble fraction, the soluble pool seemed to level off. However, the size of the soluble pool continued to increase after the initial leveling off (Figure 20).

(b) Kinetics of turnover in TCA soluble and insoluble fractions: When cells, which had been labeled for 12 hr, were washed and resuspended in medium containing 16.5 μ moles non-radioactive choline/ml

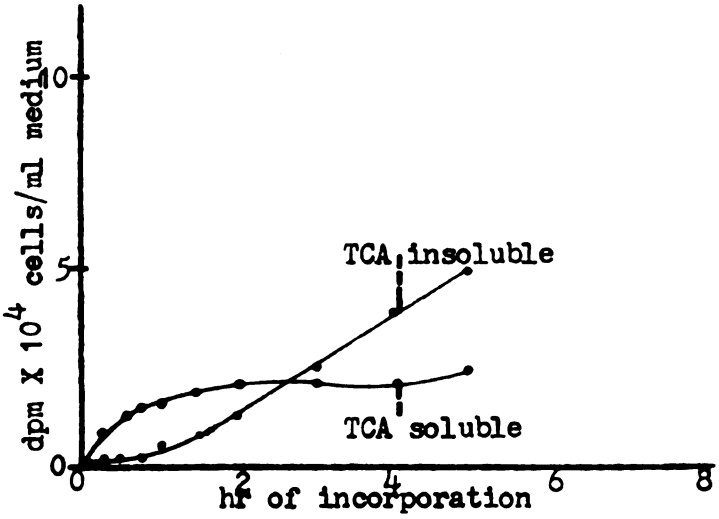

Figure 19. Kinetics of incorporation into TCA fractions. (Expt. 1)

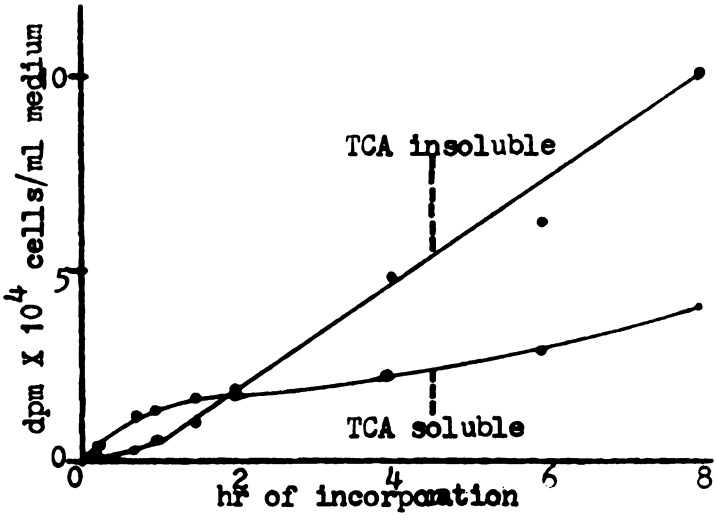

Figure 20. Kinetics of incorporation into TCA fractions. (Expt. 2)

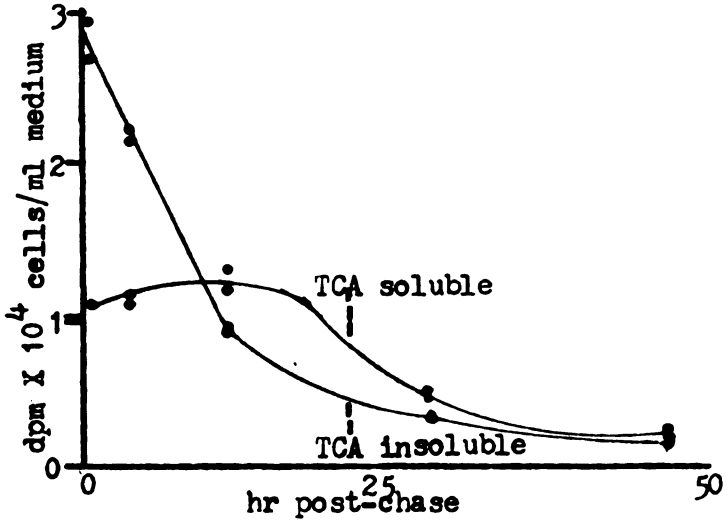

Figure 21. Kinetics of turnover in TCA fractions. (500X chase)

(500X chase), radioactivity was rapidly lost from the TCA insoluble fraction (Figure 21). Since a stable label for membrane phospholipids was being sought, the rapid turnover of this label was investigated. A population of cells, which had been labeled for 12 hr, was devided into 3 equal volume groups, washed and resuspended in medium containing 0.033 μ moles/ml (equal chase), 3.3 μ moles/ml (100X chase) and 16.5 μ moles/ml (500X chase) of non-radioactive choline (Figures 22, 23 and 24). To make the comparison more meaningful, the values are presented as percentages of the maximum TCA insoluble radioactivity. It can be seen, that as the concentration of non-radioactive choline was increased, the rate of loss of radioactivity from the TCA insoluble fraction increased.

Cell Free Incorporation and Turnover: Vandor and Richardson (1968) reported that etiolated pea seedling microsomes would incorporate C^{14}-ethanolamine, C^{14}-serine and C^{14}-choline into phospholipids. In light of the results with living Acanthamoeba, it became important to determine whether a similar system was operational. Microsomes, mitochondria and post-microsomal supernatant were prepared as described in the methods. Figure 25 illustrates cell free incorporation as a function of protein concentration. As found by Vandor and Richardson (1968), only the membrane-containing fractions (i.e. mitochondria and microsomes) incorporated C^{14}-choline. Incorporation increased as a function of protein concentration. A divalent cation requirement (Ca^{++}) is illustrated by the low level of incorporation in the presence of EDTA. Chromatography of lipid extracts revealed that all of the radioactivity was incorporated specifically into phosphatidyl choline.

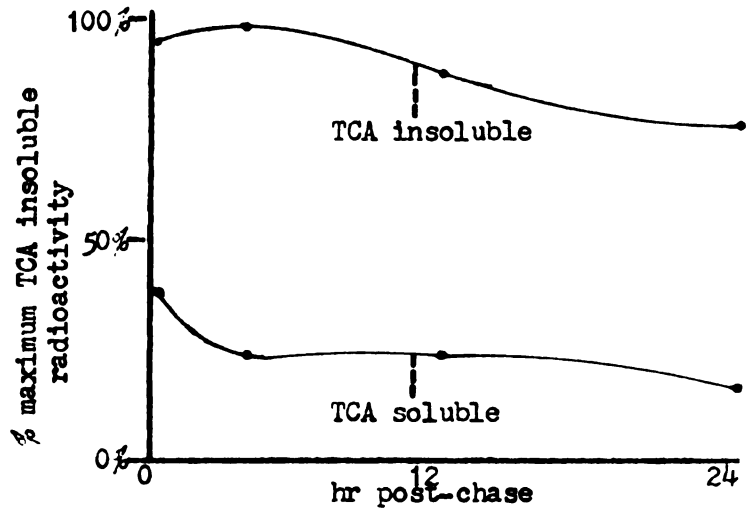

Figure 22. Kinetics of turnover in TCA fractions. (equal chase)

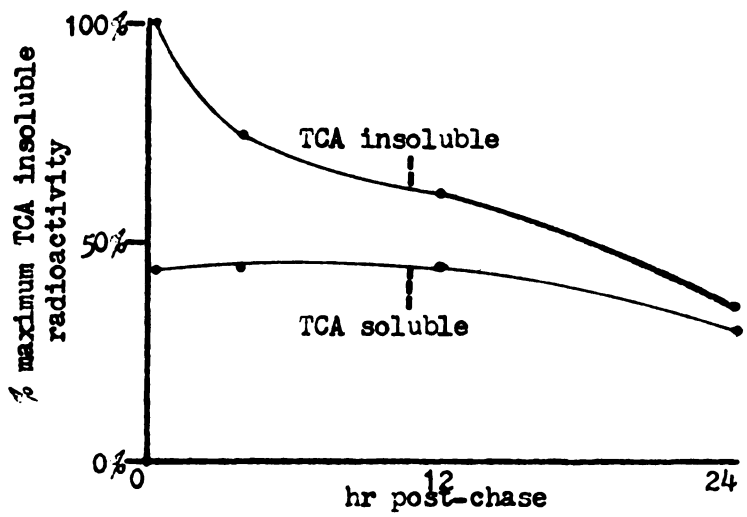

Figure 23. Kinetics of turnover in TCA fractions. (100X chase)

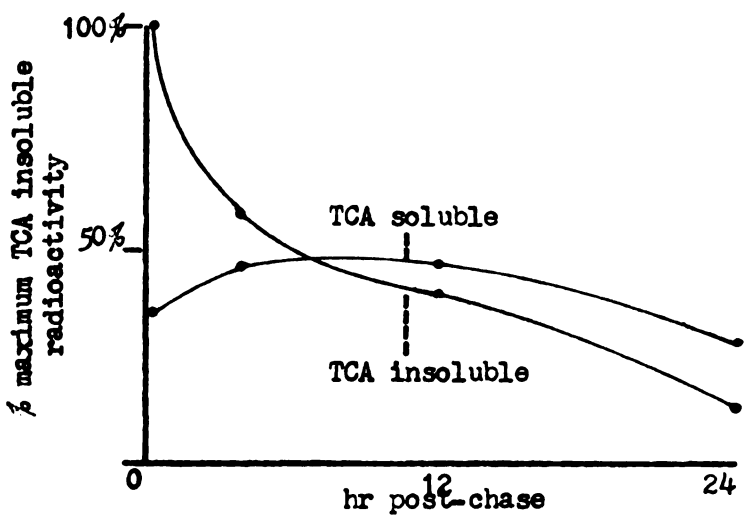

Figure 24. Kinetics of turnover in TCA fractions. (500X chase)

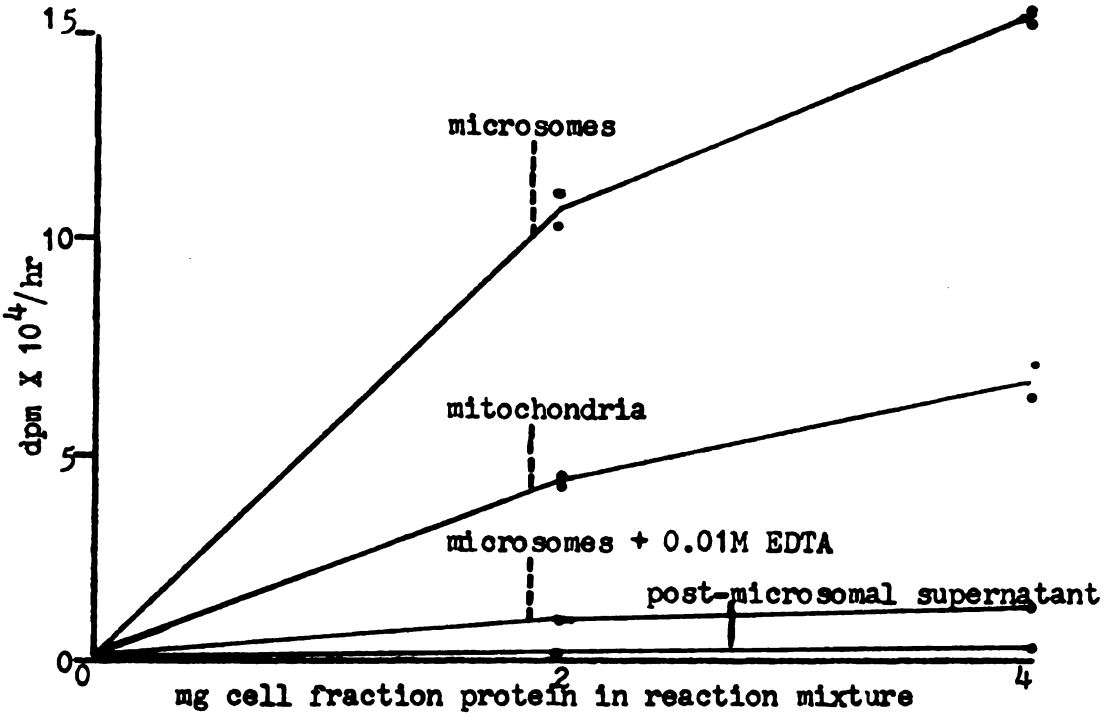

Figure 25. Cell free incorporation into cell fractions as a function of protein concentration.

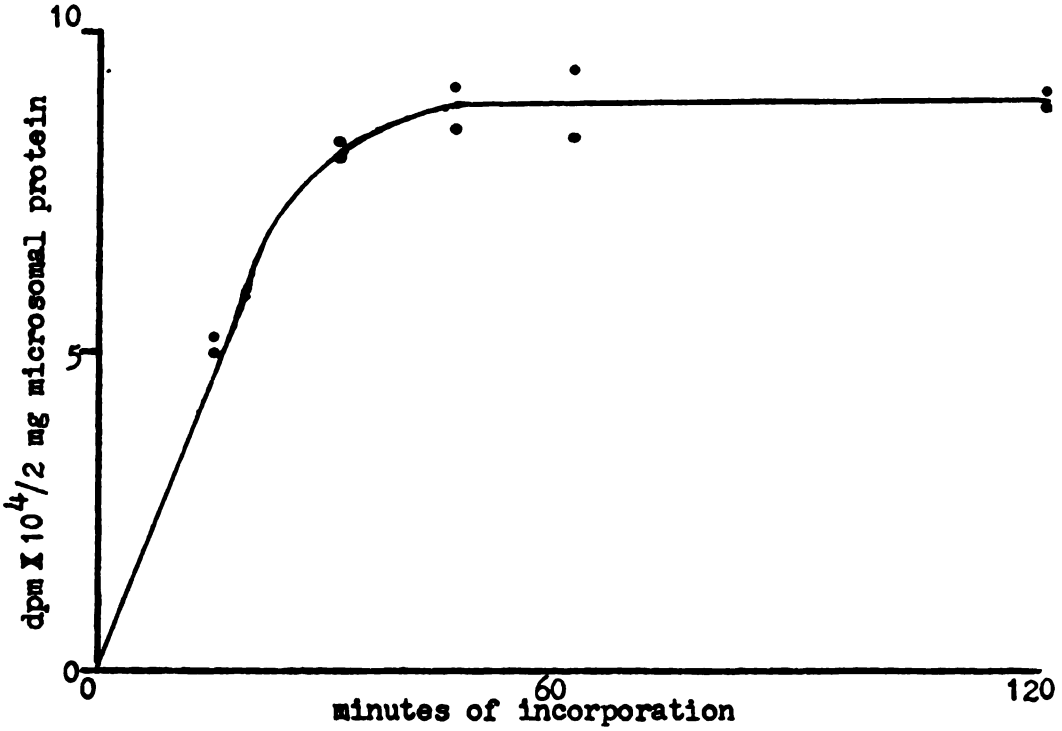

Figure 26. Cell free incorporation into microsomes as a function of time.

Figure 26 demonstrates that cell free incorporation into microsomes was initially linear as a function of time. Between 30 and 45 min, incorporation leveled off and exhibited no increase or decrease thereafter. Figure 27 illustrates that the addition of concentrated non-radioactive choline, either before or after the leveling off, not only stopped incorporation, but effected a rapid turnover of label in the cell free microsomes. Thus, an equilibrium situation seemed to be the cause of the leveling off of incorporation in the cell free system.

Comparison of Cell Radioactivity to Medium Radioactivity: To determine whether an equilibrium condition was reached by living cells and the free choline in the medium, the cell radioactivity was compared to the medium radioactivity. As explained in the methods section, cell radioactivity was the total radioactivity of washed cells from 1 ml of medium; and medium radioactivity was the total radioactivity of 1 ml of medium from which cells had been removed by centrifugation. Figures 28 and 29 demonstrate that about 70 hr after the introduction of 0.033 μ moles/ml or 3.3 μ moles/ml of non-radioactive choline, an equilibrium between cell radioactivity and medium radioactivity was reached. Figures 28 and 29 also illustrate that the attainment of this equilibrium coincided with the leveling off of the loss of lipid extractable cell radioactivity. The results have been converted to percentages of maximum cell radioactivity.

Turnover of C^{14}-Choline in Phosphatidyl Choline of Cell Fractions: In order to observe the effects of chase on the choline moiety of phosphatidyl choline in cell fractions, cells were labeled and resuspended in medium containing 3.3 μ moles/ml of non-radioactive choline

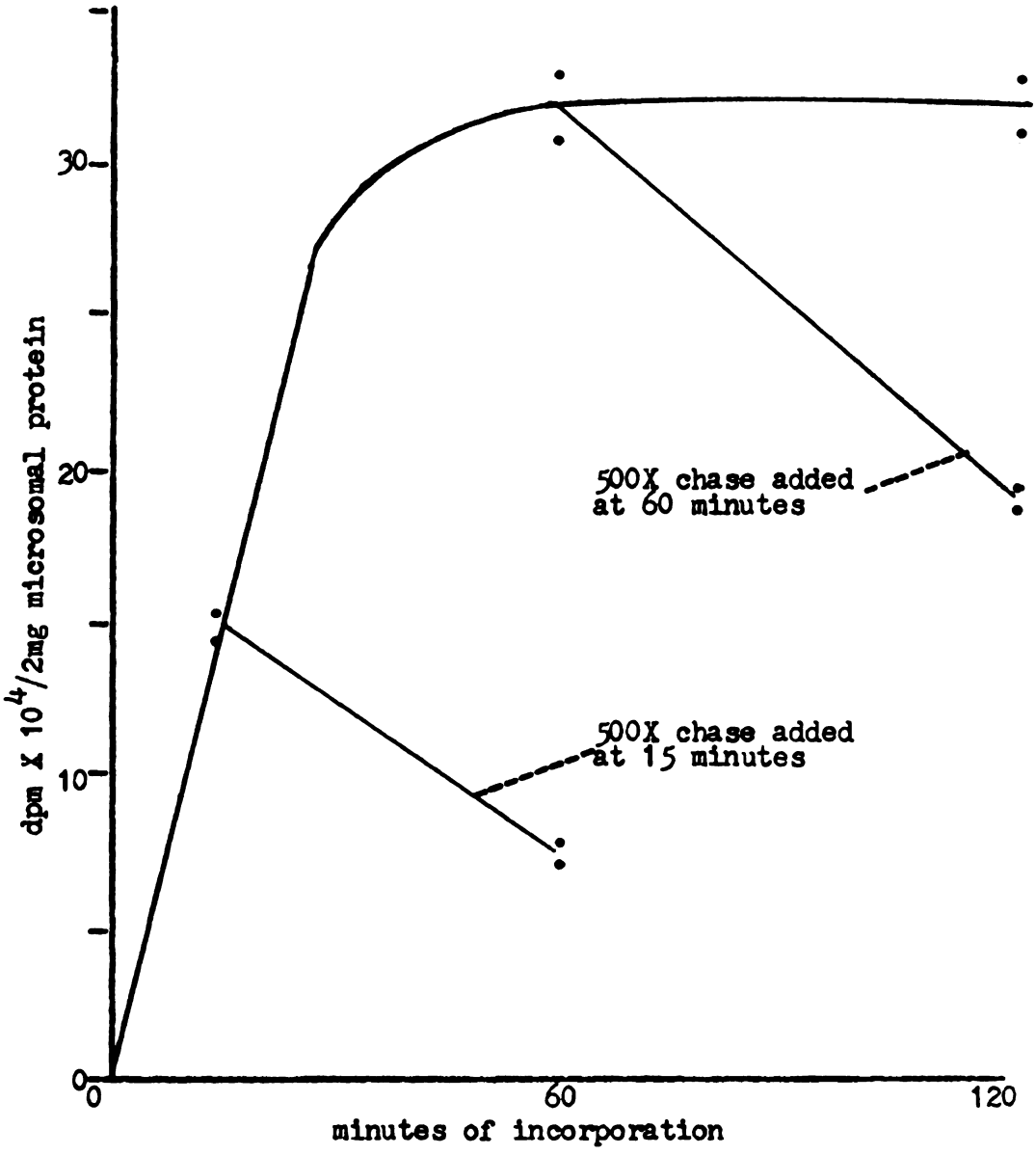

Figure 27. Cell free incorporation into microsomes as a function of time and the effects of chase.

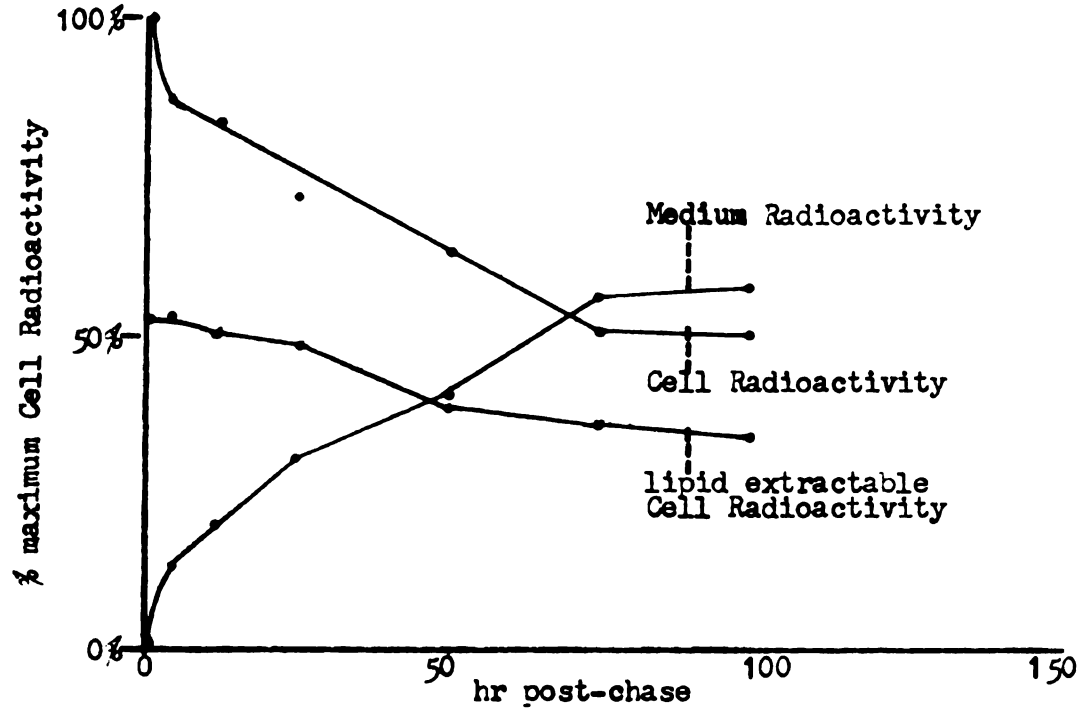

Figure 28. The effects of equal chase on Cell Radioactivity, Medium Radioactivity and lipid extractable Cell Radioactivity.

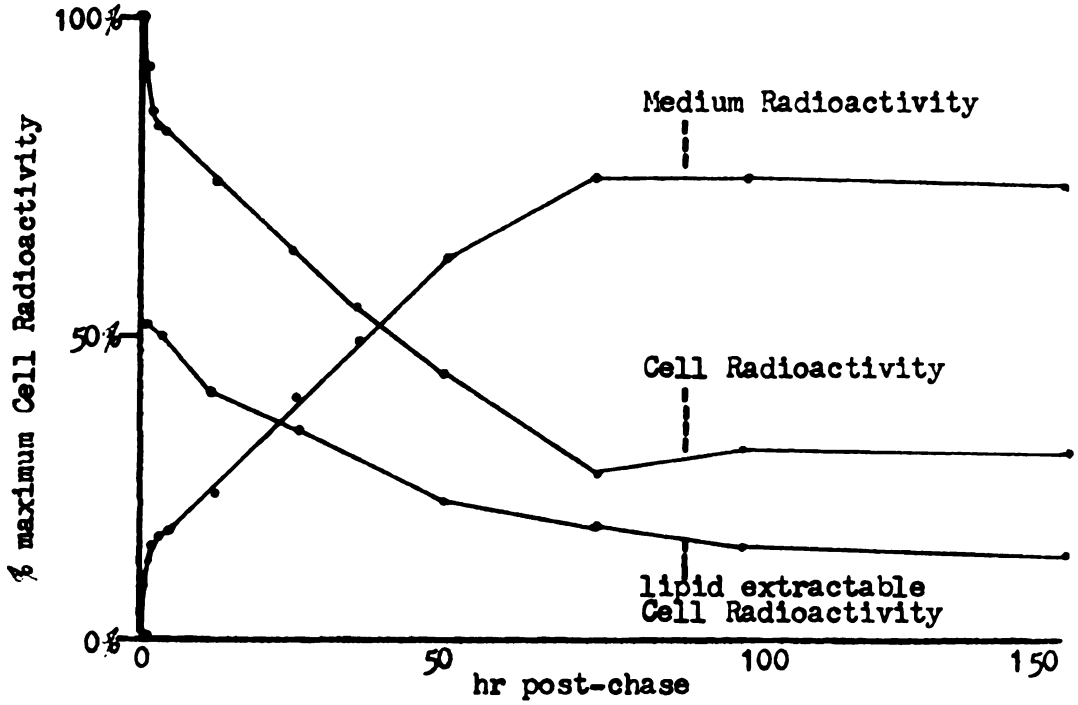

Figure 29. The effects of 100X chase on Cell Radioactivity, Medium Radioactivity and lipid extractable Cell Radioactivity.

as described. The results, summarized in Figure 30, illustrates that 2 equilibrium situations occurred. As anticipated, at about 60 to 70 hr post-chase, the loss of radioactivity from all cell fractions leveled off and the decrease in specific activities thereafter was due to growth dilution. Thus, an equilibrium was reached between free choline and the bound choline moiety. The other equilibrium attained was that reached among the cell fractions. Within 24 hr post-chase, all the cell fractions' specific activities had reached a stable position relative to each other, and decreased thereafter at the same rate. The non-membrane phosphatidyl choline in the post-microsomal supernatant also showed this equilibration effect.

Therefore, although choline was found to be a specific label for phosphatidyl choline, a peculiar exchange reaction occurred, making it an unsuitable label for studying membrane assembly.

EXPERIMENTS WITH H^3-GLYCEROL:

Specificity of Labeling:

(a) Measurement of the radioactivity of medium with growing cells: The total radioactivity of medium with growing cells did not change over a 3 day experimental period. This indicated that no radioactivity was lost from the medium into the atmosphere.

(b) Recovery of radioactivity in lipid extracts: Table 5 gives the results of 3 independent experiments on cells which had been grown for 3 days in 2.5 μc/ml of H^3-glycerol. The results indicate that essentially all of the TCA insoluble radioactivity was present in lipids.

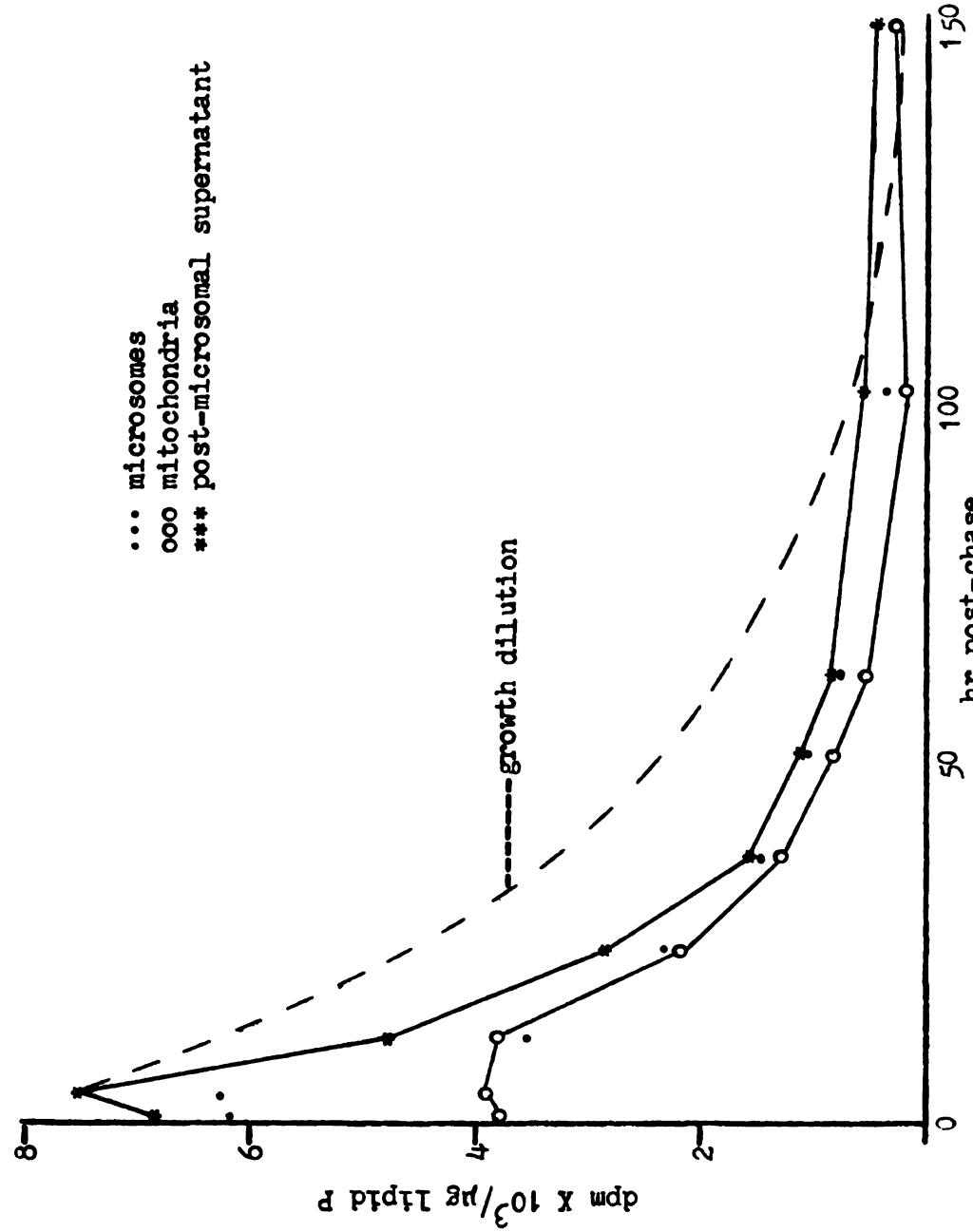

Figure 30. Turnover of C^{14}-choline in phosphatidyl choline of cell fractions.

Table 5. Recovery (%) of H^3-radioactivity in lipid extracts of cells.*

Expt. #	Lipid Extract of TCA Ppt.	Extracted TCA Ppt.	% Recovery
1	1,360,000	59,500	95.6%
2	1,242,000	43,000	96.5%
3	1,206,000	51,000	95.8%

*values given in dpm

(c) Chromatography of radioactive lipid extracts: The lipid extracts of cells incubated for 12 hr in medium containing 2.5 μc/ml of H^3-glycerol were chromatographed (see Figure 18). The radioactivity of the spots of 2 experiments are given in Table 6. All detectable phospholipids demonstrated radioactivity. The large amount of radioactivity

Table 6. Localization of H^3-glycerol in phospholipids by chromatography.*

Expt. #	PC	PI	PS	PE	SF
1	1,933	61	1,488	2,400	7,200
2	2,822	415	2,765	3,730	11,540

*values given in dpm; see Table 4 for symbol abbreviations.

recovered in the spot at the solvent front was not unexpected, since this spot should have contained triglycerides, diphosphatidyl glycerol and phosphatidic acid (Hack, Yaeger and McCaffery, 1962; Halevy and Finkelstein, 1965; Halevy, Avivi and Katan, 1966).

Since essentially all of the radioactivity of the TCA precipitatable material of cells was in lipid, direct counting of TCA insoluble fractions was used to give an approximation of the H^3-glycerol present in glycerides.

Characteristics of H^3-Glycerol Incorporation and Turnover:

(a) Kinetics of incorporation into TCA soluble and insoluble fractions: As in the C^{14}-choline experiments, values were determined on a basis of TCA soluble or insoluble radioactivity of cells from 1 ml of medium. Figures 31 and 32 illustrate that H^3-glycerol linear incorporation into lipids began almost immediately after introduction of 5 μc/ml into the medium. In addition, the size of the soluble pool was very small and showed no increase over time.

(b) Kinetics of turnover in TCA soluble and insoluble fractions: In light of the effects of varying concentrations of non-radioactive choline on the stability of the choline moiety of phosphatidyl choline, the effects of different concentrations of non-radioactive glycerol on the turnover of H^3-glycerol were studied. Cells were labeled, washed and resuspended in medium containing 0.03 μ moles/ml or 3.3 μ moles/ml of non-radioactive glycerol. The results of 2 experiments for each non-radioactive glycerol concentration are given in Figures 33 and 34. Values were converted to percentages of the maximum TCA insoluble radioactivity to aid in the comparisons. As can be seen from the graphs (Figures 33 and 34), about a 10% loss in radioactivity occurred within the first 4 hr post-chase. Thereafter, the decrease in radioactivity was slight. No effect of the different concentrations of non-radioactive glycerol was observed. The size of the soluble pool

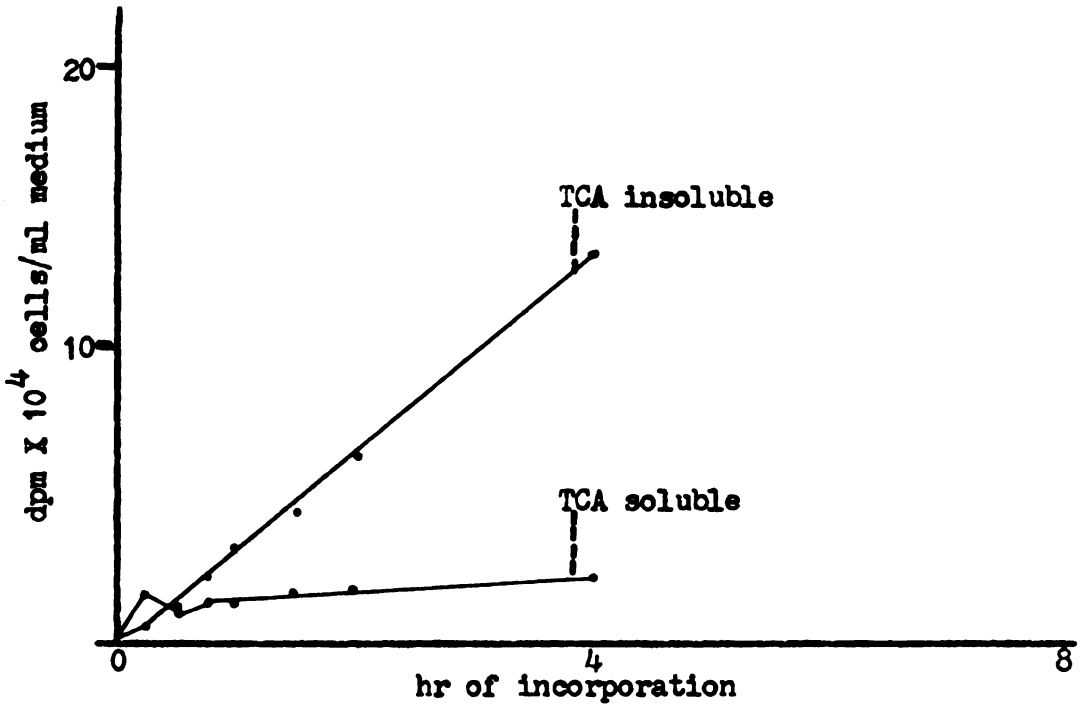

Figure 31. Kinetics of incorporation into TCA fractions. (Expt. 1)

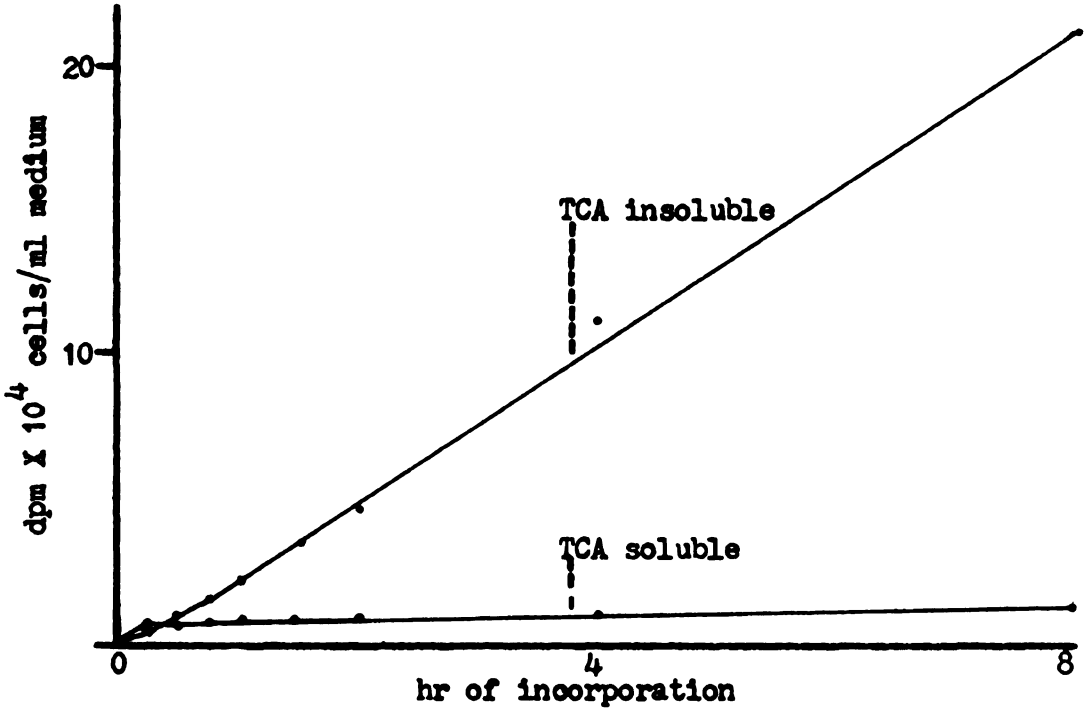

Figure 32. Kinetics of incorporation into TCA fractions. (Expt. 2)

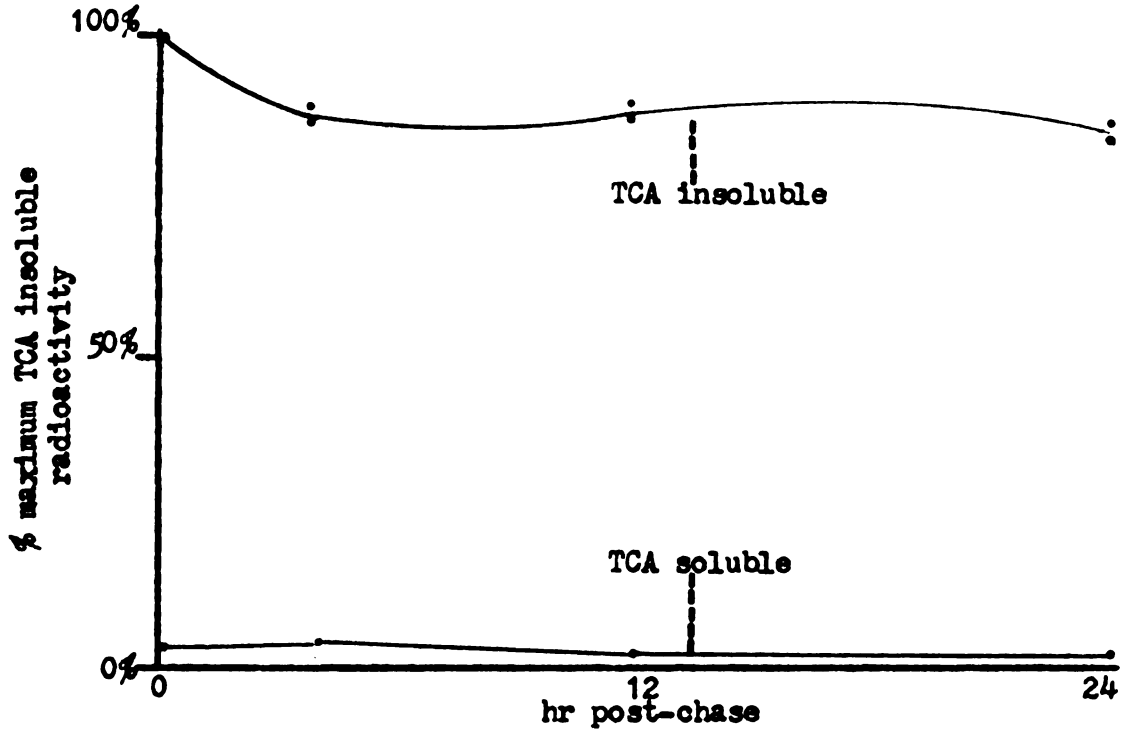

Figure 33. Kinetics of turnover in TCA fractions. (6.6X chase)

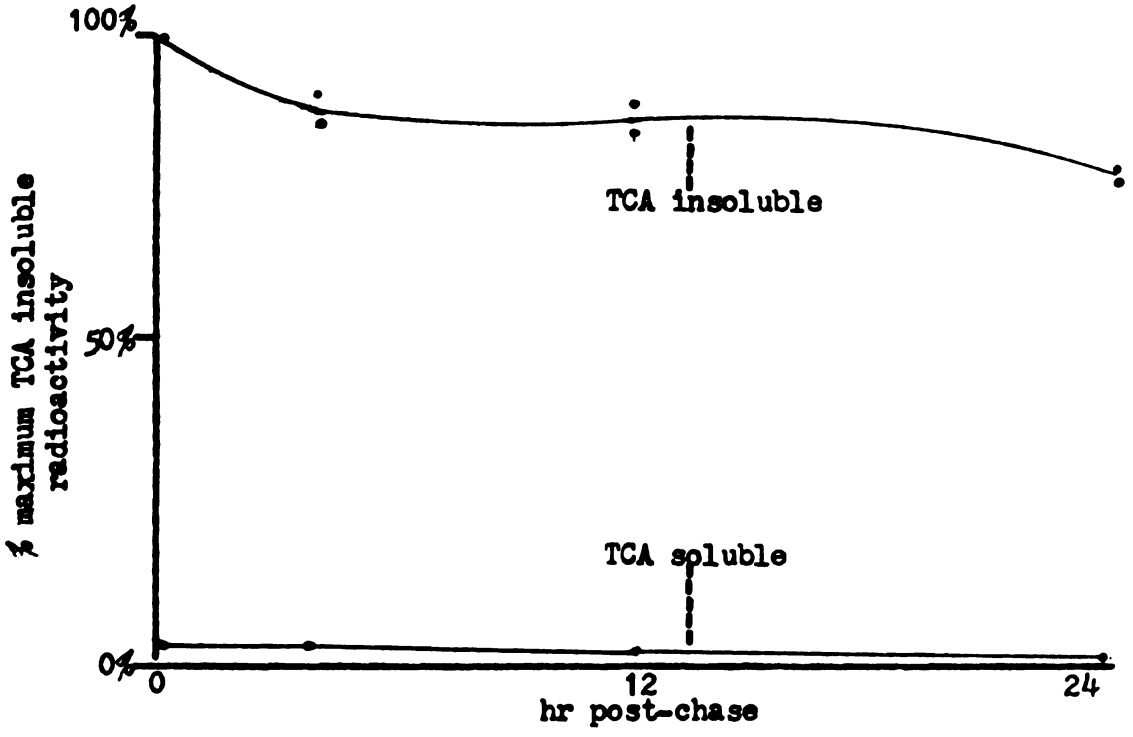

Figure 34. Kinetics of turnover in TCA fractions. (660X chase)

was extremely small.

Cell Free Incorporation: Cell fractions, prepared exactly as described for the C^{14}-choline experiments, demonstrated no cell free incorporation. Therefore, data from all of the preceding experiments with H^3-glycerol indicate that it is a relatively stable label for lipids.

Incorporation of H^3-Glycerol into Lipids of Cell Fractions: Before experiments were considered utilizing specific lipid activities of the cell fractions, it was important to determine whether the specific activities of the organelles bounded by membranes (i.e. mitochondria and nuclei) were equivalent to the particulate (i.e. membrane) or non-particulate (i.e. non-membrane) lipids of these organelles. To test this, nuclei and mitochondria from cells labeled for 12 hr in 2.0 µc/ml of H^3-glycerol were sonicated and washed in distilled water. This procedure should have removed the non-membrane lipids. The specific activities of the sonicated and washed nuclei and mitochondria were about the same as the activities of the whole nuclei and mitochondria (Table 7).

Table 7. Comparison of the specific activities of whole mitochondria and nuclei to the particulate matter of mitochondria and nuclei.*

Fraction	Particulate	Whole	% Difference
Nuclei	13,210	12,980	2%
Mitochondria	9,670	9,430	2%

*values given in dpm/µg lipid P

In the studies of incorporation of H^3-glycerol into the lipids of cell fractions, cells were fractionated at time intervals after the inoculation of H^3-glycerol according to the scheme in Figure 1. Figures 35, 36 and 37 show the pattern of incorporation over a 2 hr period. Incorporation into the non-membrane lipids of the post-microsomal supernatant was linear and occurred at a more rapid rate than incorporation into any of the other cell fractions. The only time this might not have been true was within the first 5 min of incorporation. Five minutes after the beginning of incorporation the nuclear and the supernatant fractions' specific activities were approximately the same. Of all the membrane fractions the nuclear fraction maintained the highest specific activity during all points of the incorporation experiments (Figures 35, 36 and 37). For this reason, the nuclear fraction's specific activities were used as a reference for relative comparisons among the specific activities of other fractions. For these comparisons, relative specific activity ratios (i.e. $\frac{\text{specific activity of fraction}}{\text{specific activity of nuclear fraction}}$) were calculated.

In the table (Table 8) of relative specific activity ratios, it can be observed that the ratios changed after 12 hr in medium containing H^3-glycerol. The ratios attained after 12 hr in labeling medium were designated as "labeling equilibrium" ratios. Those values accepted as the "labeling equilibrium" ratios were those that were the same (i.e. differed less than $\pm$ 0.1 from the values of the other 2 experiments) in 2 out of the 3 experiments (see 0 time in Figures 38, 39 and 40). As can be seen in Table 8, only 1 "labeling equilibrium" value of the elongate endoplasmic reticulum and 1 value for the

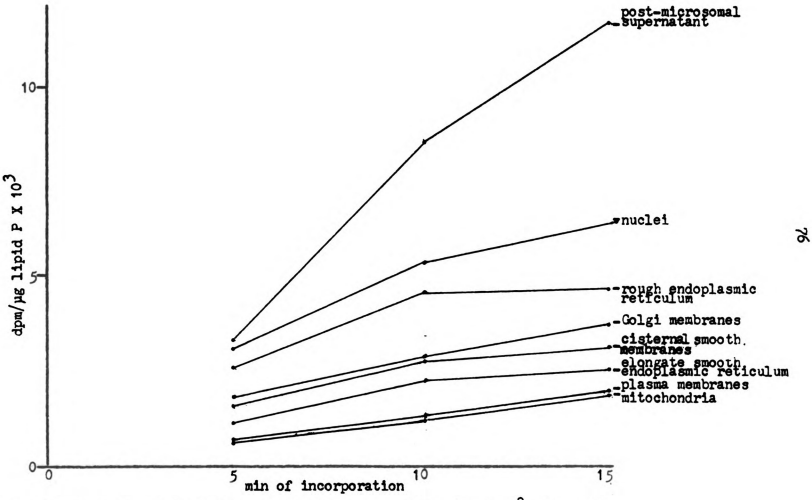

Figure 35. Incorporation into lipids of cell fractions. (40 µc/ml of H^3-glycerol)

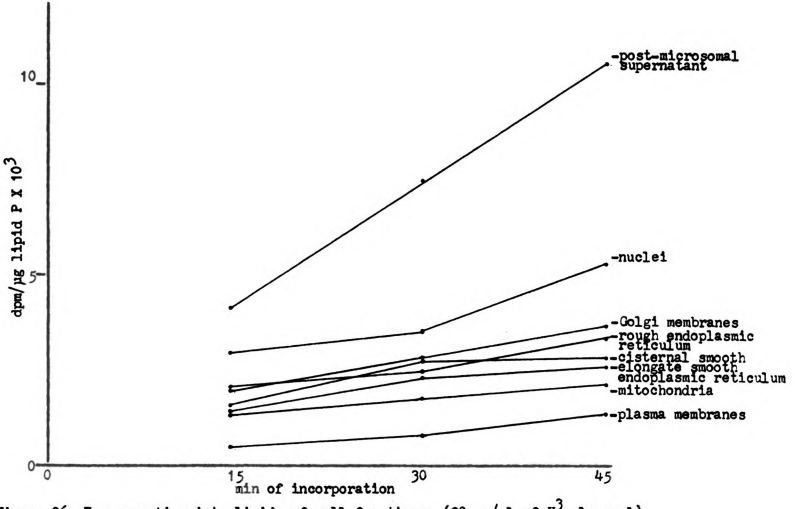

Figure 36. Incorporation into lipids of cell fractions. (20 µc/ml of H^3-glycerol)

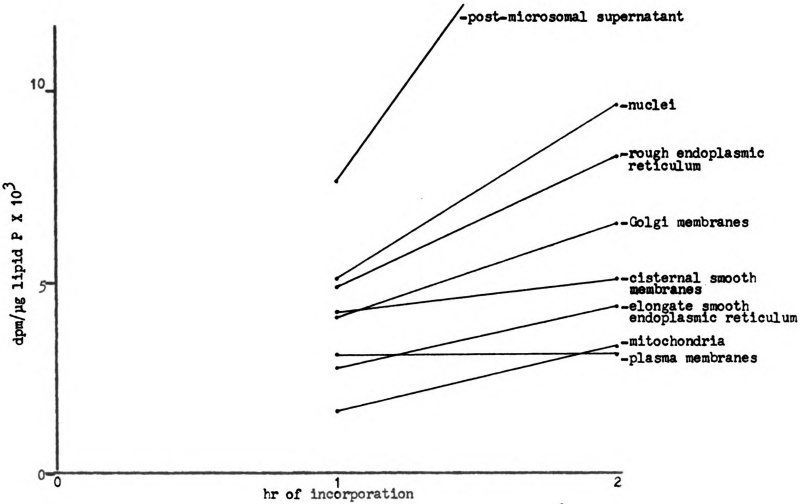

Figure 37. Incorporation into lipids of cell fractions. (20 µc/ml of H^3-glycerol)

Table 8. Comparison of relative specific activity ratios ($\frac{\text{specific activity of fraction}}{\text{specific activity of nuclear fraction}}$) at temporal intervals after the inoculation of radioactive glycerol.*

Fraction	5 min (Fig. 35)	2hr (Fig. 37)	"labeling equilibrium ratios" 12 hr (Fig. 38)	12 hr (Fig. 39)	12 hr (Fig 40)
N	1	1	1	1	1
RER	0.83	0.87	0.90	0.93	0.86
C-SM	0.50	0.52	0.75	0.72	0.75
PDM	0.20	0.31	0.66	0.54**	0.74
G	0.59	0.68	0.64	0.70	0.70
E-SER	0.36	0.45	0.83**	0.68	0.68
M	0.19	0.33	0.72**	1.00**	0.56**
PMS	1.10	2.21	12.65	11.98	3.00

*symbol definitions are given in Table 2.
**ratios differ by more than 0.1 from the other 2 experimental ratios at the end of 12 hr period

plasma and digestive vacuole membrane fraction were not in agreement and are marked. The mitochondrial fraction demonstrated no "labeling equilibrium" position.

Within the first 5 min of incorporation, the rough endoplasmic reticulum was in its "labeling equilibrium" position (Table 8). By 2 hr the Golgi fraction had also reached its highest relative specific activity. Sometime between 2 and 12 hr after the introduction of radioactive glycerol into the medium, the plasma and digstive vacuole membranes, the cisternal smooth membranes and the elongate smooth endoplasmic reticulum reached their "labeling equilibrium" positions.

Turnover of H^3-Glycerol in Lipids of Cell Fractions: The primary objective of the turnover studies was to determine the relative changes in specific activities of the cell fractions. Thus, no corrections for growth dilution are shown on the graphs (Figures 38 and 39). The cells were growing exponentially in these experiments. The changes in specific activities of fractions of cells, which had been labeled in 2.0 μc/ml of H^3-glycerol for 12 hr, washed and resuspended in medium containing 3.3 μ mole/ml of non-radioactive glycerol, are shown in Figures 38 and 39. Table 9 gives the actual decrease over the 24 hr chase period in percentages. The expected decrease due to growth dilution would have been 44%.

The nuclear and the rough endoplasmic reticulum fractions decreased at a more rapid rate than any of the other fractions. The non-membrane lipids of the post-microsomal supernatant decreased at a rate slower than all of the other fractions. Table 10 illustrates the relative specific activity ratios after the 24 hr period. In contrast to the

Table 9. Decrease (%) in specific activity over a 24 hr chase period.*

Fraction	Figure 38	Figure 39
N	70	74
RER	76	67
C-SM	52	66
PDM	43	61
G	53	62
E-SER	57	57
M	52	62
PMS	20	43

*symbol definitions are given in Table 2.

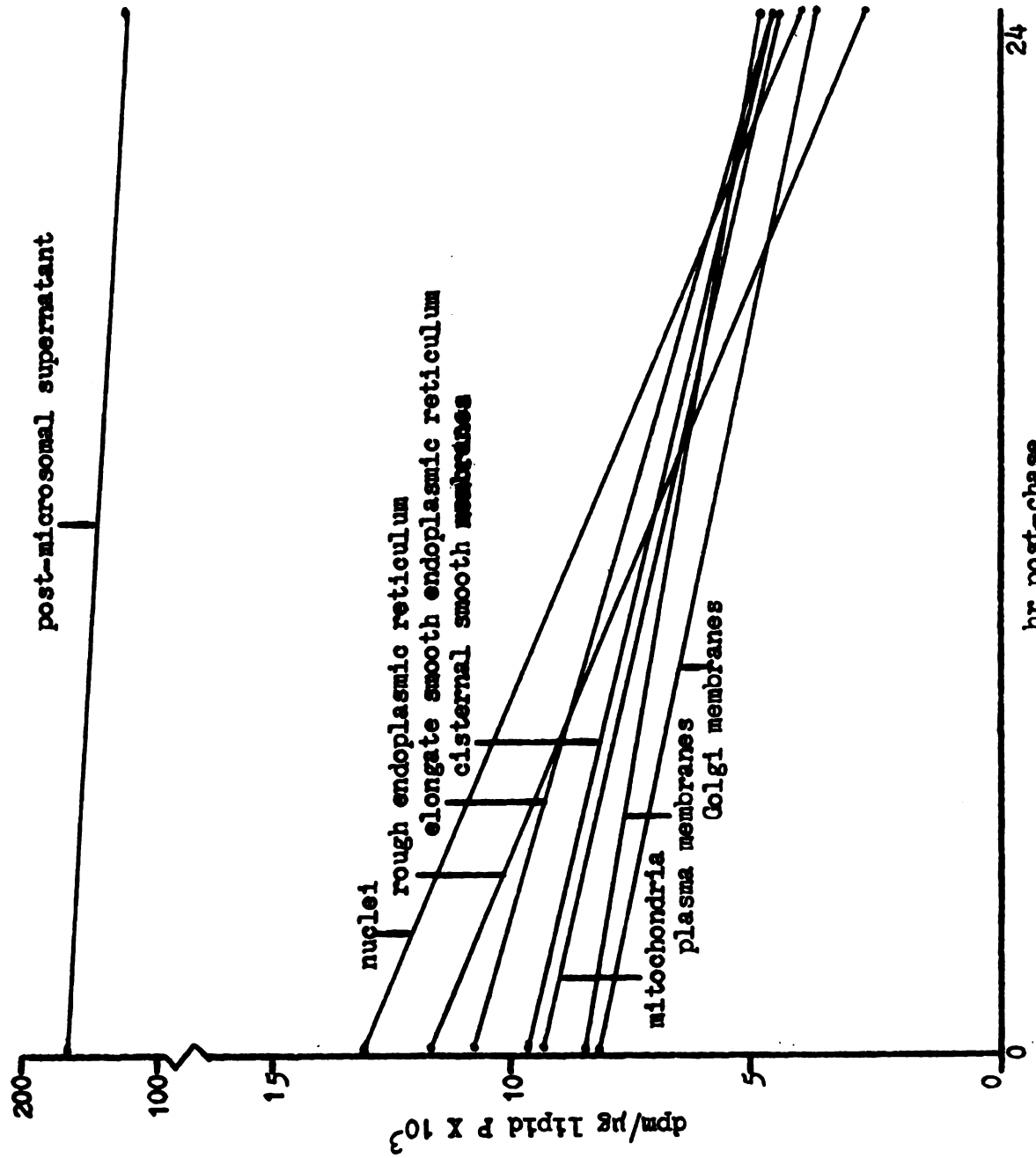

Figure 38. Turnover of H^3-glycerol in lipids of cell fractions. (Expt. 1)

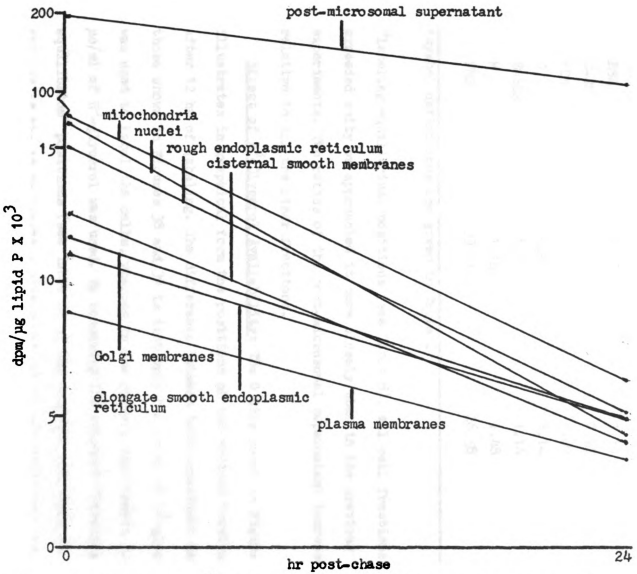

Figure 39. Turnover of H^3-glycerol in lipids of cell fractions. (Expt. 2)

Table 10. Relative specific activity ratios ($\frac{\text{specific activity of fraction}}{\text{specific activity of nuclear fraction}}$) at the end of a 24 hr chase period.*

Fraction	Figure 38	Figure 39
N	1	1
RER	0.72	1.19
C-SM	1.18	0.93
PDM	1.23	0.80
G	0.99	1.14
E-SER	1.18	1.14
M	1.16	1.48
PMS	33.05	26.58

*symbol definitions are given in Table 2.

"labeling equilibrium" positions (see Table 8), all cell fractions exceeded unity or approached it more closely than in the previous experiments. The ratios of the post-microsomal supernatant increased relative to all the other fractions.

Effect of H^3-Glycerol Availability: The 0 time point in Figure 40 illustrates in graphical form the positions of the various fractions after 12 hr of labeling. The difference between this experiment and those shown in Figures 38 and 39 is that only 0.2 μc/ml of H^3-glycerol was used to label the cells, whereas in the other 2 experiments 2.0 μc/ml of H^3-glycerol was used. By comparing the relative "labeling equilibrium" positions (see Table 8) of the membrane fractions at the end of the 12 hr labeling period, it can be seen that they are

all the same with the qualifications previously mentioned. However, the non-membrane lipids of the post-microsomal supernatant in this experiment (Figure 40) has a very low ratio when compared to the other 2 experiments (Figures 38 and 39). In Table 11, using the nuclear

Table 11. Comparison of 12 hr labeling specific activities of nuclear and post-microsomal supernatant fractions.*

Fraction	Figure 40 (0.2 μc/ml)	Figure 38 (2.0 μc/ml)	Figure 39 (2.0 μc/ml)
Nuclei	4,380	12,980 (3X)	16,120 (4X)
Post-Microsomal Supernatant	12,940	164,100 (13X)	194,000 (15X)

*values given in dpm/μg lipid P; values in parenthesis show increase over value in Figure 40.

fraction as a representative of the "labeling equilibrium" positions of the membrane fractions, it can be seen that the increased amount of radioactive glycerol available in the medium, increased the specific activity of the non-membrane lipids to a greater extent than the membrane's lipid specific activity. In both cases, the "labeling equilibrium" of the membrane fractions is unaffected by the amount of radioactive glycerol available (see Table 8).

Pulse and Chase of H^3-Glycerol in Lipids of Cell Fractions: Figure 40 ("long pulse" experiment) illustrates the effects of a 3.3 μ moles/ml non-radioactive glycerol "chase" on cells which had been labeled for 12 hr in medium containing 0.2 μc/ml of H^3-glycerol.

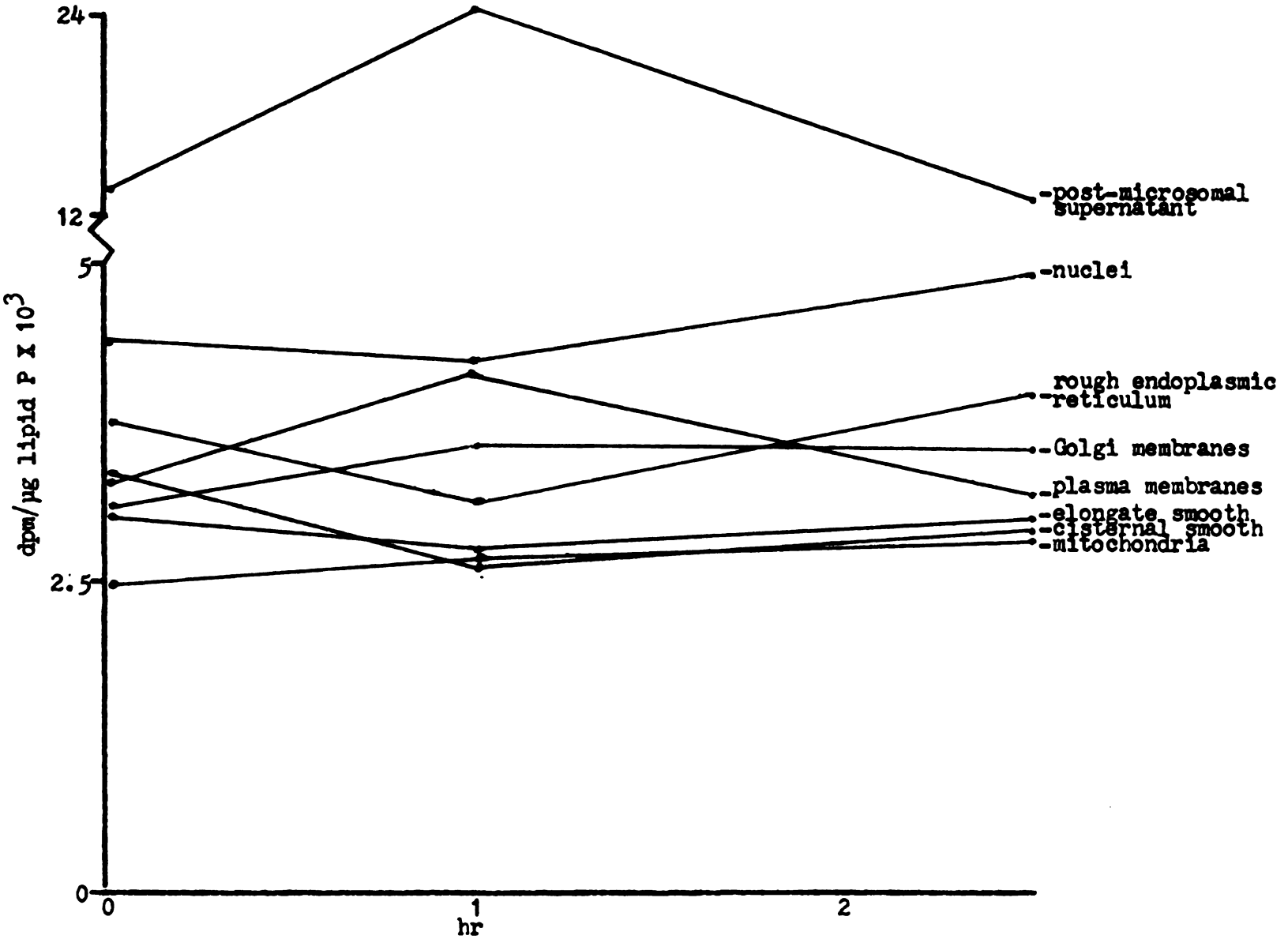

Figure 40. "Long pulse" experiment. Cells labeled for 12 hr prior to being washed and chased in medium containing 3.3 µ moles/ml of non-radioactive glycerol.

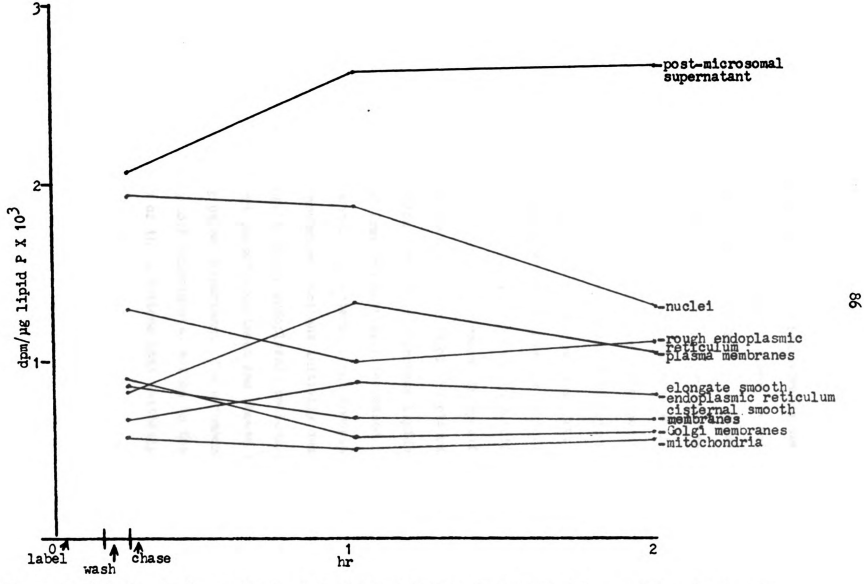

Figure 41. "Short pulse" experiment. Cells labeled for 10 min prior to being washed and chased in medium containing 3.3 µ moles/ml of non-radioactive glycerol.

Figure 41 ("short pulse" experiment) demonstrates the effects of the same concentration of non-radioactive glycerol on cells which had been labeled for 10 min in medium containing 20 μc/ml of H^3-glycerol. There is one major difference between the two experiments. In the "long pulse" experiment, all the fractions were well labeled and had reached their "labeling equilibrium" positions (see Table 8). This obviously was not true in the "short pulse" experiment. With the latter fact in mind, it may be predicted that some fractions would be affected by the chase differently in these two experiments.

The Golgi membranes initially decreased in specific activity in the "short pulse" experiment, and increased in specific activity in the "long pulse" experiment. The lipids of the plasma and digestive vacuole membrane fraction and the non-membrane lipids of the post-microsomal supernatant fraction initially increased following the chase in both experiments. The nuclear, rough endoplasmic reticulum and cisternal smooth membrane fractions initially decreased in both experiments. The elongate smooth endoplasmic reticulum initially increased in the "short pulse" experiment and showed a slight initial decrease in the "long pulse" experiment. The mitochondrial fraction simply leveled off in both experiments. Notice in the "long pulse" experiment, that many of the fractions that initially decreased in the first hr post-chase, increased in the next time interval.

DISCUSSION

CELL FRACTIONATION:

Fractions Isolated: In fixed cells, membranes occur in morphologically and, most probably, in functionally distinct locations and configuration. Rough surfaced membranes are found as part of the nuclear envelope and as the rough endoplasmic reticulum in the cytoplasm. Smooth membranes can be observed in the form of plasma membranes, vacuole membranes, Golgi complex membranes, smooth endoplasmic reticulum, mitochondrial membranes, and cisternal smooth membranes in Acanthamoeba (Bowers and Korn, 1968; this paper).

Previous fractionation procedures have been developed for the isolation of a particular cell component or, at most, several cell components, from the same tissue or group of cells (e.g. Blobel and Potter, 1966; deDuve, Pressman, Gianetto, Wattiaux and Appelmans, 1955; Hogeboom, Schneider and Palade, 1948; Jamieson and Palade, 1967a; Schneider and Kuff, 1954). Smooth surfaced and rough surfaced microsomes have been separated on the basis of density differences (e.g. Rothschild, 1961; Dallner, Siekevitz and Palade, 1966a). Subfractionation of these microsomal membranes were devised utilizing deoxycholate solubilization (Ernster, Siekevitz and Palade, 1962), cation effects on sedimentation rates (Dallner and Nilsson, 1966) and density gradient centrifugation of rough endoplasmic reticulum elements (Dallner, Bergstrand and Nilsson, 1968). All of the latter procedures dealt with small membrane vesicles produced by homogenization and

fractionation, rather than intact membrane systems (see Dallner and Ernster, 1968, for a review).

More often then not, the parameters of isolation procedures were adjusted in such a way that many of the organelles or membrane systems not being isolated, were destroyed in the course of fractionation. Since the eventual goal of the present research was the study of temporal events during membrane biogenesis, it was necessary that as many as possible of the membrane-bound organelles and membrane systems be isolated from the same cell homogenate. Furthermore, an attempt was made to isolate the structurally distinct membrane systems intact. In this manner, the complications of trying to subfractionate a group of homogeneous-looking vesicles was avoided. In order to prevent vesiculation of the membrane systems and organelles, it was observed that careful control had to be exercised over homogenization, isolation media and sedimentation. The intact cell components finally isolated were: (1) nuclei with intact envelopes, (2) rough endoplasmic reticulum, (3) elongate smooth endoplasmic reticulum, (4) small, cisternal smooth membranes, (5) Golgi complex membranes, (6) plasma and digstive vacuole membranes, (7) mitochondria and (8) post-microsomal supernatant with the non-membrane lipids of the cell. The only major membrane-bound elements not isolated were lysosomes and peroxisomes.

It has been reported (Bowers and Korn, 1968) that the smooth endoplasmic reticulum in Acanthamoeba may occur in the form of tubular elements. Observations on fixed cells used in this study confirmed the latter report (Figure 5). These tubular membranes, as well as

large cisternal membranes, were recovered after gradient centrifugation in band 1 (Figure 7). The digstive vacuole membranes were observed to sediment with the plasma membrane fragments. This is not suprising in view of the fact that the digestive vacuoles are derived directly from the cell surface membranes (e.g. Korn and Weisman, 1967). Lysosomes and peroxisomes, which normally sediment in the micochondrial fraction (deDuve, 1967), were not apparent ultrastructurally. Presumably, these organelles were removed from the mitochondrial fraction by the extensive low-speed washings (Band and Morhlok, 1969). With the exception of the mitochondria, all of the fractions illustrated good morphological preservation and a relatively high degree of purity.

Chemical Analysis: The RNA/protein ratios (Table 1) of the elongate type of smooth endoplasmic reticulum and the rough endoplasmic reticulum are in substantial agreement with published values of microsomes equilibrating at the same densities (Omura, Siekevitz and Palade, 1967). The presence of RNA in the small, cisternal smooth membrane fraction and the Golgi membrane fraction correlated with the ultrastructural observations that free ribosome-like particles and rough endoplasmic reticulum vesicles, respectively were present in these fractions (Figures 13, 14, 15 and 16). The negative chemical tests for RNA in the plasma and digestive vacuole membrane fraction also was in accord with electron microscope observations (Figure 10).

Whole cells,as well as microsomes, contained phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol (Figure 18). Triglycerides, diphosphatidyl glycerol and sterols

also have been reported in Acanthamoeba (Hack, Yaeger and McCaffery, 1962; Halevy and Finkelstein, 1965; Halevy, Avivi and Katan, 1966).

Enzyme Analysis: Glucose-6-phosphatase is normally associated with microsomes in certain mammalian organs (Swanson, 1955). The specific activity of this enzyme in the plasma and digstive vacuole membrane fraction was the highest observed. Some activity was also present in the Golgi and rough endoplasmic reticulum fractions. The presence of glucose-6-phosphatase activity in the mitochondrial fraction indicated a microsomal contaminant or activity in the mitochondrial membranes.

Thiamine pyrophosphatase is normally used as a marker for Golgi membranes and Golgi associated membranes (Novikoff, Essner, Goldfischer and Heus, 1962). Very little of this enzyme was soluble (Table 2). High specific activities were found in the plasma and digstive vacuole membrane fraction, the Golgi membrane fraction and the small, cisternal smooth membrane fraction. The rough and elongate smooth endoplasmic reticulum contained a smaller, but considerable, amount of activity.

Magnesium-dependent adenosine triphosphatase activity was ubiquitously distributed among the membrane-containing fractions (Table 2). This was not unexpected, since this enzyme has been found in practically all membranes (Novikoff, Essner, Goldfischer and Heus, 1962). What was suprising was the lack of stimulation of these activities in the plasma membrane-containing fraction by sodium and potassium ions. Sodium and potassium-stimulated, magnesium-dependent adenosine triphosphatase is often used as a marker for cell surface membranes (Novikoff, Essner, Goldfischer and Heus, 1962). The lack of monovalent

cation stimulation of the magnesium-dependent adenosine triphosphatase might have been due to the fact that the plasma and digestive vacuole membrane fraction was isolated in a medium containing a high potassium content (0.025M KCl). In contrast to the findings with glucose-6-phosphatase and thiamine pyrophosphatase, 0.25M sucrose-TKM washings had no deleterious effect upon adenosine triphosphatase activities in the plasma and digestive vacuole membrane fraction. It is possible that the values observed with magnesium-dependent adenosine triphosphatase already include a cation stimulation. An alternative explanation would be that there is no sodium and potassium-stimulated, magnesium-dependent adenosine triphosphatase in the plasma membranes of Acanthamoeba. Klein (1964) observed that oubain, the classical inhibitor of cation transport and sodium and potassium-stimulated, magnesium-dependent adenosine triphosphatase, had no effect on potassium transport in Acanthamoeba.

The enzyme analysis data (Table 2) indicated that the enzyme activity in the plasma and digestive vacuole membrane fraction was enriched for every phosphatase examined. It is not known whether the membrane-bound enzymes reside specifically in the plasma membranes, the digestive vacuole membranes or both.

A CHOLINE EXCHANGE REACTION:

Cell Free Incorporation and Turnover: The amounts of C^{14}-choline incorporated into phosphatidyl choline increased as a function of mitochondria or microsome protein concentration (Figure 25). No incorporation into the non-membrane lipids of the post-microsomal

supernatant fraction was observed, suggesting that membranes are the site of this reation. Incorporation of C^{14}-choline into the phosphatidyl choline of microsomes was linear as a function of time up to about 30 min, before leveling off (Figure 26). Vandor and Richardson, (1968), reporting on a virtually identical system in etiolated pea seed "microsomal suspensions", offered 3 possible alternative reasons for the leveling off of incorporation: (1) depletion of an essential cofactor or substrate, (2) attainment of equilibrium by the reaction or (3) inactivation of the enzyme involved.

As was demonstrated, addition of concentrated non-radioactive choline into the cell free system before or after the leveling off period, resulted in a rapid turnover of label (Figure 27). Thus, an equilibrium had been reached, where the amount of radioactive choline exchanging onto and off the microsomal phosphatidyl choline was the same. As in Vandor and Richardson's (1968) experiments, a Ca^{++} requirement was indicated. The latter authors also gave evidence that the pea seed cell free system involved a single enzyme.

Incorporation of choline into the membranes of isolated rat liver mitochondria has been reported (Kaiser and Bygrave, 1968; Bygrave and Kaiser, 1968). However, this was not a direct exchange reaction and required optimal concentrations of ATP and CTP, indicating a synthetic pathway. Although a choline exchange reaction also has been reported in rat liver microsomes (Dils and Hübscher, 1961), recent attempts to duplicate this finding have been unsuccessful (Nagley and Hallinan, 1968; Stein and Stein, 1969).

A Choline Exchange Reaction in Living Cells: The data reported indicated that a choline exchange reation also occurred in living cells. If the concentrations of non-radioactive choline were increased in turnover experiments, the loss of radioactivity from phosphatidyl choline was increased (Figures 22, 23 and 24). Therefore, the turnover of the choline moiety of phosphatidyl choline was a function of the concentration of non-radioactive choline. Upon comparing cell radioactivity to medium radioactivity in turnover experiments, it was found that an equilibrium was eventually reached between the cell radioactivity and the medium radioactivity (Figures 28 and 29). The position of that equilibrium was dependent upon the concentration of non-radioactive choline in the medium. With a larger concentration of non-radioactive choline, more radioactivity was lost from the cells into the medium before leveling off occurred. Thus, in both the cell free and the living cell experiments, the exchange reaction was a function of the concentration of non-radioactive choline and reached an equilibrium.

In the living cells, the attainment of an equilibrium coincided with the leveling off of loss of lipid extractable cell radioactivity (Figures 28 and 29). Extending this to observations on cell fractions, the decrease in specific activities of cell fraction lipids also reached an equilibrium at the same time (Figure 30). In addition, it was observed that within 24 hr post-chase, all the cell fractions' specific activities reached a stable position relative to each other. The non-membrane phosphatidyl choline of the post-microsomal supernatant also showed this equilibration. If, in the living cells, as was

true in the cell free system, choline exchange only occurred with membrane-bound phosphatidyl choline, then the non-membrane phosphatidyl choline must somehow exchange with membrane phosphatidyl choline, before its choline moiety is exchanged. If this did not happen, the maximum decrease in specific activity that could be expected, would be that due to growth dilution (see Figure 30). Therefore, either the non-membrane phosphatidyl choline of the supernatant fraction exchanged (not necessarily a direct exchange) with membrane phosphatidyl choline and had its choline moiety exchanged, or in contrast to the cell free system, a choline exchange reaction occurred in non-membrane phosphatidyl choline. It has been reported (Wirtz and Zilversmit, 1968) that phospholipids, but not proteins, exchange between liver mitochondria and microsomes in vitro. It is possible, that an exchange between non-membrane and membrane phospholipids occurs in living Acanthamoeba.

LIPID SYNTHESIS AND ASSEMBLY OF LIPIDS INTO MEMBRANES:

Specificity and Stability of H^3-Glycerol as a Lipid Label: The results reported, indicated that H^3-glycerol was a specific label for phospholipids and other glycerides (Table 6). Unlike C^{14}-choline, variations in the concentration of non-radioactive glycerol had no effect on the rate of H^3-glycerol turnover in lipids (Figure 33 and 34). In fact, glycerol-containing lipids exhibited a high degree of stability. For these reasons, H^3-glycerol was found to be a suitable label for studying the sites of synthesis of glycerol-containing lipids and assembly of these lipids into membranes.

Incorporation of H^3-Glycerol into Lipids of Cell Fractions:

Incorporation of H^3-glycerol into the non-membrane lipids of the post-microsomal supernatant was linear and occurred at a rate faster than that found in any of the other cell fractions (Figures 35, 36 and 37). Of all the membrane fractions, incorporation was most rapid into the lipids of nuclear membranes and rough endoplasmic reticulum. The relative specific activity ratios in Table 8 illustrate that the rough endoplasmic reticulum was in its "labeling equilibrium" position at the earliest time measured (5 min). Of all the smooth membranes, the Golgi membranes were the only ones to reach their "labeling equilibrium" position within the first 2 hr of incorporation. The other smooth membrane fractions reached "labeling equilibrium" positions between 2 and 12 hr after incubation in H^3-glycerol-containing medium. The mitochondria, which incorporated radioactivity into their lipids at a slow rate, did not seem to reach a stable specific activity position relative to the other membranes.

Since incorporation was most rapid in the post-microsomal supernatant's non-membrane lipids, this location must be either a site of lipid synthesis or an area where newly synthesized lipids are rapidly transferred. The nuclear membranes and rough endoplasmic reticulum are implicated as sites of synthesis of lipids and assembly of these lipids into membranes. This would agree with the finding that newly synthesized phospholipids are incorporated more rapidly into rough surfaced microsomes, then smooth surfaced microsomes, in rat liver (Dallner, Siekevitz and Palade, 1966a). The Golgi membrane fraction did not incorporate radioactivity as quickly as the post-microsomal supernatant,

nuclear membranes and rough endoplasmic reticulum; but the Golgi membrane fraction did reach its "labeling equilibrium" within the first 2 hr of incorporation (see Table 8). Thus, it is likely that these membranes did not synthesize lipids, but did incorporate lipids derived from another location at a faster rate than the other smooth membrane fractions, or incorporated at the same rate as the other smooth membrane fractions, but turned over faster. The elongate smooth endoplasmic reticulum, the small, cisternal smooth membrane and the plasma membrane fractions might be membranes which derive presynthesized lipids from another fraction. Since the micochondria seem to be independent of the other membrane systems with respect to a "labeling equilibrium" position, these organelles might synthesize and utilize their own membrane lipids, derive their lipids from another source or both (Stein and Stein, 1969).

Effects of the Availability of H^3-Glycerol on "Labeling Equilibrium" Positions: The specific activity of the non-membrane lipid was found to be greatly affected by the concentration of radioactive glycerol used to label the cells for 12 hr, whereas the membrane fractions were not (see Tables 8 and 11). Using the nuclear fraction as representative of the membranes (Table 11), it can be seen that a 10-fold increase of the amount of H^3-glycerol in the medium, resulted in an increase in membrane specific activity of about 3X. On the other hand, the specific activity of the non-membrane lipid increased 13X to 15X. The "labeling equilibrium" positions of the membrane fractions were unaffected by the concentration of glycerol available (Table 8). It seems logical, that if membrane lipids were derived from the

non-membrane lipid, a more rigid relationship between the specific activities of the non-membrane and membrane lipids would be maintained. The given results would imply that membrane lipids are not derived from the pool of lipids available in the non-membrane lipid fraction.

Turnover of H^3-Glycerol Labeled Lipids in Cell Fractions: Over a 24 hr chase period (Figures 38 and 39) the non-membrane lipids of the post-microsomal supernatant decreased in specific activity at a slower rate than any other fraction (Table 9). The relative specific activity ratios (Table 10) of the non-membrane lipids increased with respect to all the membrane fractions. All the membranes decreased at a faster rate than that expected due to growth dilution (Table 9). Thus, it seems that lipid radioactivity lost from the membranes goes to the non-membrane lipids of the post-microsomal supernatant fraction. Of all the membranes, activity was lost most rapidly from the nuclear membranes and rough endoplasmic reticulum (Table 9). This correlates with the results of the incorporation studies and again suggests that these 2 fractions might be sites of lipid synthesis and incorporation of lipids into membranes. The relative specific activity ratios (Table 10) of all the other membrane fractions increased with respect to the nuclear fraction.

Omura, Siekevitz and Palade (1967) and Widnell and Siekevitz (1967) have reported that the glycerol moiety of phospholipids in rough and smooth microsomes, nuclear membranes and plasma membranes of rat liver turnover at the same rate. There are several distinctions between the

latter authors experiments and those being presented. First, in adult liver, cells are not growing and there is a question as to whether one is studying newly elaborated membranes, molecular replacement in stable membranes, or both. Secondly, and most importantly, in the liver cell studies, no measurements were made before 23 hr post-injection of the isotope. In this manner, any pattern of equilibration of lipid radioactivity through, for instance, recycling of membranes or membrane lipids would have been missed. In the experiments being considered, the cells were growing and an elaboration of new membranes was assured. Turnover did seem to be apparent, with loss of lipids from the membranes to the non-membrane fraction. In the Acanthamoeba experiments, the exact rates of turnover were not being measured, but the relative changes in specific activity ratios were, and indicated that the nuclear membranes and the rough endoplasmic reticulum were probable sites of lipid synthesis and assembly of lipids into membranes.

Pulse and Chase of H^3-Glycerol Labeled Lipids in Cell Fractions: In both the "long" and the "short pulse" experiments (Figures 40 and 41) the non-membrane lipids of the post-microsomal supernatant continued to increase in specific activity in the first hr post-chase. This, along with the results of the labeling experiments (Table 11) and the turnover experiments (Figures 38 and 39) is evidence that the non-membrane lipids are synthesized in and derived from membranes. In the second half of the "short pulse" experiment the specific activity leveled off and in the second part of the "long pulse" experiment the specific activity decreased. This would imply that the non-membrane

lipids were finally being diluted by chase phopholipids, transfering some of their lipids back to the membranes or some combination of these 2 events. Several factors argue against, but do not rule out, reutilization of non-membrane lipids in membranes. First, the equilibrium ratios of the membrane fractions were not affected by the amount of radioactive glycerol available (Tables 8 and 11). Secondly, over a 24 hr chase period (Figures 38 and 39; Table 10) radioactivity was lost from the membranes to the non-membrane lipid.

In the first hr immediately following both the "long" and the "short pulse" experiments, the nuclear membranes' specific activities leveled off. This would imply that if the nuclear membranes are sites of lipid synthesis and incorporation of lipids into membranes, these membranes are not transferred away from the nucleus very rapidly. Another alternative would be that the nucleus rapidly receives membranes with lipids synthesized elsewhere or non-membrane lipids, and the chase effect is somewhat masked. In the second hr of the "short pulse" experiment, the nuclear membranes rapidly decreased in specific activity. However, in the second half of the "long pulse" experiment, the nuclear membranes increased in specific activity. Thus, a source of presynthesized lipids for the nuclear membranes is "hot" in the "long pulse" experiment and "cold" in the "short pulse" experiment. This source might be either recycled membrane lipids or non-membrane lipids. In both the "short" and the "long pulse" experiments the non-membrane lipids' specific activity was much higher than the nuclear membrane specific activity; but it was only in the "long pulse"

experiment that all the membranes were well labeled. Thus, it would have to be suggested that the post-chase increase, in the nuclear membranes specific activity in the second half of the "long pulse" experiment, was due to recycling of membrane lipids.

The rough endoplasmic reticulum decreased rapidly in specific activity in the first hr following the chase in both the "short" and the "long pulse" experiments. This fact, together with all the other experimental data to this point, pinpoints this location as the major lipid synthesizing site in the cell. In addition, because the specific activity decreased rapidly, the lipids in the rough endoplasmic reticulum must be rapidly transferred elsewhere. The newly synthesized lipids could be transferred as non-membrane lipids, in the form of membranes, or both. In the second half of both the "long" and the "short pulse" experiments, the rough endoplasmic reticulum increased in specific activity. Again, a recycling of lipids is apparent.

The small, cisternal smooth membranes decreased in specific activity in the first hr of both the "long" and the "short pulse" experiments. The incorporation evidence does not implicate this type of membrane as a site of lipid synthesis. If it is not a site of lipid synthesis, then it must be an area which either rapidly incorporates newly sysnthesized lipids, rapidly transfers these lipids elsewhere or a combination of these events. In the second hr of the "short pulse" experiment, the specific activity of this fraction leveled off. In the second part of the "long pulse" experiment this fraction increased slightly in specific activity. Again, a recycling effect is possible.

The Golgi membranes' specific activity decreased in the first hr of the "short pulse" experiment and increased in the first hr of the "long pulse" experiment. From the incorporation data, it was suggested that the Golgi membranes were not sites of lipid synthesis but were sites which incorporated newly synthesized lipids. If, in addition, its membranes were rapidly turning over (i.e. transferring these lipids elsewhere), this would fit into the rapid decrease in specific activity observed in the first part of the "short pulse" experiment. Since it increased in specific activity initially, following the chase in the "long pulse" experiment, its source or sources of membrane lipids remain "hot" for a considerable time following the chase. This fraction's specific activity leveled off in the second half of the "short pulse" experiment and decreased rapidly in the second part of the "long pulse" experiment. One could envision that this was when the chase effect was finally felt, but since it was observed that other fractions were actually increasing in specific activity at this time, a recycling effect is more probable, in addition to the chase effect.

Of all the membranes, the plasma and digestive vacuole membranes incorporated radioactivity the slowest (Figures 35, 36 and 37), even though their eventual "labeling equilibrium" position was higher than, for example, the Golgi membranes (Table 8). This fact, coupled with the knowledge that the plasma membranes continued to increase in both the "long" and the "short pulse" experiments strongly suggests that these membranes are derived from another membrane type.

The fact that the elongate type of endoplasmic reticulum increased in the first part of the "short pulse" experiment would suggest that

these membranes, like the plasma membranes were receiving presynthesized membranes. However, their decrease in specific activity in the first part of the "long pulse" experiment seems to contradict this conclusion, and at present can not be explained.

Once again, the mitochondria seem to be the exceptions. In both the "long" and the "short pulse" experiments, this fraction simply levels off. Therefore, mitochondria either synthesize their own lipids, utilize newly synthesized lipids, or both (cf. Stein and Stein, 1969). The mitochondrial fraction's specific activity did not decrease at a rate much different than any of the other membranes in the turnover experiments (Figures 38 and 39; Table 9). This would suggest, that although the modes of incorporation are different, the rates of turnover are about the same.

Conclusions: The rough endoplasmic reticulum is probably the major site of phospholipid and glyceride synthesis in Acanthamoeba palestinensis. The nuclear membranes might also be sites of lipid synthesis, but most certainly are sites where labeled membranes rapidly appear. All the other membrane fractions seem to receive presynthesized lipids, possibly in the form of membranes. The post-microsomal supernatant's non-membrane lipids are mostly derived from newly synthesized membrane lipids. However, lipids from membrane turnover also seem to be transferred to this fraction. Since no membrane fraction completely lost its radioactivity in the turnover and the pulse and chase experiments, one would have to predict a reutilization or recyling of membrane lipids. Cyclic patterns observed in the pulse and chase experiments, suggest that there is a recycling of membrane lipids. It seems likely,

particularly in the case of the plasma and digestive vacuole membranes, that this recycling occurs within the structure of the membranes. However, in most of the cases, it can not be stated with certainty that this recycling of membrane lipids involves the transformation of one membrane type to another, although it is strongly suggested. Most of the evidence does indicate that this recycling does not involve the non-membrane lipids. It will be interesting once the glycerol studies are complete, to study proteins in the same fashion. A correlation of the glycerol experiments with some other membrane label is necessary to discover whether recycling occurs by selective exchange of membrane lipids, or by interconversions of one membrane type to another.

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