ASSEMBLY OF LIPIDS INTO MEMBRANES IN *ACANTHAMOEBA PALESTINENSIS*

I. Observations on the Specificity and Stability of Choline-^{14}C and Glycerol-^{3}H as Labels for Membrane Phospholipids

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ABSTRACT

In order to determine the feasibility of using radioactive precursors as markers for membrane phospholipids in *Acanthamoeba palestinensis*, the characteristics of phospholipids labeled with choline-^{14}C and glycerol-^{3}H were examined. Choline-^{14}C was found to be a specific label for phosphatidyl choline. There was a turnover of the radioactive moiety of phosphatidyl choline at a rate that varied with the concentration of nonradioactive choline added to the growth medium. Radioactivity was lost from labeled phosphatidyl choline into the acid-soluble intracellular pool and from the pool into the extracellular medium. This loss of radioactivity from cells leveled off and an equilibrium was reached between the label in the cells and in the medium. Radioactive choline was incorporated into phosphatidyl choline by cell-free microsomal suspensions. This incorporation leveled off with the attainment of an equilibrium between the choline-^{14}C in the reaction mixture and the choline-^{14}C moiety of phosphatidyl choline in the microsomal membranes. Therefore, a choline exchange reaction may occur in cell-free membranes, as well as living *A. palestinensis*. In contrast to choline-^{14}C, the apparent turnover of glycerol-^{3}H–labeled phospholipids was not affected by large concentrations of nonradioactive choline or glycerol in the medium. The radioactivity in lipids labeled with glycerol-^{3}H consisted of 33% neutral lipids and 67% phospholipids. Phospholipids labeled with glycerol-^{3}H turned over slowly, with a concomitant increase in the percentage of label in neutral lipids, indicating a conversion of phospholipids to neutral lipids. Because most (~96%) of the glycerol-^{3}H recovered from microsomal membranes was in phospholipids, whereas only a minor component (~2%) of the glycerol-^{3}H was in the phospholipids isolated from nonmembrane lipids, glycerol-^{3}H was judged to be a specific marker for membrane phospholipids.

INTRODUCTION

Because phospholipids are a ubiquitous component of biological membranes, radioactive phospholipid precursors are often used to label membranes (1, 2, 3, 4, 5, 6, 7). As a prelude to studies of membrane phospholipids in the soil ameba, *Acanthamoeba* (=*Mayorella*) *palestinensis* (8, 9), we ex-

amined the specificity and stability of choline-^{14}C as a marker for phosphatidyl choline and glycerol-^{3}H as a marker for phospholipids in membranes. Other investigators have reported exchange reactions of choline (10), serine (11, 12), ethanolamine (13, 14, 15), and inositol (16) in cell-free systems, but the existence of these reactions in vivo has been questioned (7, 17). Recently, new evidence has been obtained in vivo for an exchange of bases between choline and phosphatidyl choline in liver (18). Data presented in this report indicate that the choline moiety of phosphatidyl choline may undergo an exchange reaction in living *A. palestinensis*, as well as in cell-free microsomal membrane suspensions. The results also indicate that glycerol-^{3}H is a specific and relatively stable label for membrane phospholipids.

Portions of this work have been reported briefly (19, 20).

METHODS

Cell Culture

A. palestinensis was cultured axenically in silicone-coated flasks containing proteose peptone-glucose medium (21). Cells were incubated at 29°C with agitation (100 rpm[1]) on a rotary shaker (22) and grew exponentially with a doubling time of 27 hr. Growth was monitored by direct counting in all experiments.

Radioactive Labeling

Choline-Me-^{14}C (7.6 μCi/μmole) and glycerol-2-^{3}H (500 μCi/μmole) were purchased from New England Nuclear Corp., Boston, Mass. Cells were grown during incorporation experiments, at an initial concentration of $\sim 10^6$ cells per milliliter, in fresh medium containing either 1 μCi choline-^{14}C/ml (132 nmoles/ml) or 5 μCi glycerol-^{3}H/ml (10 nmoles/ml). For turnover experiments, cells were grown for 12 hr in medium supplemented with either 0.25 μCi choline-^{14}C/ml (33 nmoles/ml) or 2 μCi glycerol-^{3}H/ml (4 nmoles/ml). At the end of the labeling period the cells were collected and washed twice in unlabeled medium by centrifugation at 125 *g* for 5 min. The washed cells were resuspended at a concentration of $\backsim 10^6$ cells per milliliter in fresh "chase" medium supplemented with 0.033, 3.3, or 16.5 μmoles nonradioactive choline chloride/ml or 16.5 μmoles nonradioactive glycerol/ml.

Radioactivity Measurements

During the course of either incorporation or turnover experiments, replicate 1 ml samples of well-mixed cell suspensions were removed and diluted 10-fold with ice-cold solution containing 0.25 M sucrose, 0.005 M Tris-HCl, and 0.002 M $MgSO_4$ (pH 7.5). The cells were harvested and washed twice in the ice-cold solution by centrifugation at 125 *g* for 5 min. The washed cells were either prepared for radioactivity determinations, as described below, or were brought to 1 ml (original volume) with ice-cold 10% trichloroacetic acid (TCA), and stored for at least 2 hr at 4°C. The acid-insoluble fraction was centrifuged for 10 min at 16,000 *g* in a Sorvall SS-34 fixed-angle rotor. Portions of the clarified, acid-soluble fraction were removed and counted directly in scintillation fluid. The acid-insoluble material was washed twice in ice-cold 5% TCA and once in distilled water by centrifugation at 16,000 *g* for 10 min to remove residual soluble radioactivity (1). The TCA-insoluble precipitate was extracted for lipids, as will be described, or was dissolved completely in 88% formic acid and transferred to scintillation vials for counting. In this manner, the acid-soluble and acid-insoluble radioactivity of cells could be related directly to the number of cells per milliliter of growth medium.

Cell radioactivity was determined by restoring the pellets of washed cells recovered from 1 ml of medium to a volume of 1 ml with distilled water, resuspending the cells and counting portions in scintillation fluid. The total radioactivity of 1 ml of medium, from which the cells had been removed by centrifugation, was ascertained by the counting of portions of that medium in scintillation fluid.

Cell Fractionation

In experiments to determine the percentage of lipids labeled with glycerol-^{3}H in microsomes and lipid droplets, an isolation medium containing 0.25 M sucrose, 0.005 M Tris-HCl, and 0.002 M $MgSO_4$ (pH 7.5) was used. To isolate microsomal fractions from unlabeled cells for cell-free reaction mixtures, the isolation medium contained 0.25 M sucrose and 0.1 M phosphate buffer at a pH of 7.4 (13).

Labeled or unlabeled cells from 150 to 200 ml of medium were harvested and washed twice in ice-cold isolation medium by centrifugation at 125 *g* for 5 min in a Sorvall HB-4 swinging-bucket rotor. Thereafter, all steps were carried out at 4°C.

The wet, packed cells were resuspended in 15 ml of isolation medium and homogenized with seven strokes of a 30 ml Potter-Elvehjem glass–Teflon grinder (Arthur H. Thomas Co., Philadelphia, Pa.)

[1] *Abbreviations used are:* dpm, disintegrations per minute; EDTA, ethylenediaminetetraacetic acid; lipid P, lipid phosphorus; PPO, 2,5-diphenyloxazole; rpm, revolutions per minute; TCA, trichloroacetic acid; bis-MSB, *p*-bis-(o-methylstyryl)-benzene.

at 2500 rpm. The homogenate was centrifuged at 10,000 *g* for 25 min in a Sorvall SS-34 fixed-angle rotor to remove nuclei, mitochondria, and cell debris. The supernatant was decanted with essentially all of the floating lipids and centrifuged for 1.5 hr at 100,000 *g* in a Spinco 30 fixed-angle rotor to yield the microsomal and postmicrosomal fractions. The top one-fourth of the postmicrosomal supernatant was decanted, taking care to recover all of the floating lipids. Presumably the floating lipids were derived mainly from lipid droplets seen in fixed cells. The remaining postmicrosomal supernatant was discarded. The lipid droplets and the microsomal pellet were resuspended separately in isolation medium and washed by centrifugation at 100,000 *g* for 1.5 hr.

In experiments where the cells had been labeled with glycerol-^{3}H, the washed microsomes and lipid droplets (floating lipid layer and top one-fourth of the postmicrosomal supernatant) were precipitated with 10% (final concentration) TCA and allowed to stand for a minimum of 2 hr at 4°C. Before lipid extraction the acid-insoluble material was washed as previously described.

Cell-Free Reaction Mixture

The cell-free system was prepared according to the method of Vandor and Richardson (13). Microsomal fractions, isolated as described above, were diluted to 1 mg protein/ml as determined by the Folin-Ciocalteau test (23), with a standard solution containing 0.821 μM $CaCl_2$ and 0.1 M phosphate buffer (pH 7.4). Either 2 or 4 ml of the suspension was brought to 5.25 ml with the standard solution to yield a mixture containing 2 or 4 mg of microsomal protein. The cell-free suspensions were allowed to warm from 4°C to 29°C in a water bath. After temperature equilibrium (~5 min), 1 μCi choline-^{14}C (132 nmoles) or 5 μCi glycerol-^{3}H (10 nmoles) was introduced into the reaction mixture in 0.25 ml of the standard solution. The complete reaction mixture contained 2 or 4 mg of microsomal protein, 132 nmoles choline-^{14}C or 10 nmoles glycerol-^{3}H, and 5 μmoles $CaCl_2$, made up to a total volume of 5.5 ml with 0.1 M phosphate buffer (pH 7.4). After incubation at 29°C, with agitation, the reactions were terminated at specified time intervals by adding equal volumes of ice-cold, 20% TCA. The TCA suspensions were stored for a minimum of 2 hr at 4°C before washing and extraction of lipids.

Lipid Isolation

The total lipids of washed TCA precipitates of cells or cell fractions were extracted twice with 10 volumes of chloroform:methanol (2:1) for 2 hr at room temperature. The extracts were washed with 0.1 N HCl to remove residual protein (24). Separate portions were used for chromatographic analysis, lipid phosphorus determination, and scintillation counting.

The pooled chloroform:methanol extracts were spotted on activated silica gel plates and developed in chloroform:methanol:acetic acid:water (25:15:4:2) (25). The separated phospholipids were detected by iodine vapors and scraped, together with the associated silica gel, into scintillation vials for counting. Alternativelv, the extracts were evaporated to dryness, and the lipids were dissolved in chloroform and separated into neutral lipids and phospholipids by column chromatography with silicic acid (26). Lipid phosphorus was determined according to Bartlett (27). Portions of lipid extract were evaporated to dryness in counting vials before the addition of scintillation fluid.

Liquid Scintillation Counting

Radioactive counting of aqueous solutions, as well as of lipid extracts evaporated to dryness in scintillation vials, was done in 15 ml of *p*-dioxane scintillation fluid containing 7.84 g/liter 2,5-diphenyloxazole (PPO), 0.16 g/liter bis-MSB and 120 g/liter naphthalene (New England Nuclear Corp.). A Packard Tri-Carb liquid scintillation counter was used to determine counting rates, which were corrected for background and quenching to disintegrations per minute (dpm).

RESULTS

Specificity of Labeling

When cells were grown in medium containing initial concentrations of 1 μCi choline-^{14}C/ml (132 nmoles/ml) or 5 μCi glycerol-^{3}H/ml (10 nmoles/ml) essentially all of the acid-insoluble radioactivity of cells was extractable in the total lipid fraction. That is, approximately 98% of the ^{14}C-radioactivity and ~95% of the ^{3}H-radioactivity was recoverable in chloroform: methanol (2:1) extracts when samples were analyzed at 1, 8, 24, and 72 hr after introduction of isotope into the medium. Apparently little, if any, choline-^{14}C or glycerol-^{3}H was converted to a nonlipid, acid-insoluble form by cells growing in proteose-peptone glucose medium.

In thin-layer chromatography (25) of the lipids of cells grown for 12 hr in medium containing 1 μCi choline-^{14}C, all of the radioactivity was localized in phosphatidyl choline. The radioactive lipids of cells labeled for 12 hr with 2 μCi glycerol-^{3}H/ml medium consisted of 33% neutral lipids and 67% phospholipids, as separated by column

chromatography (26). Therefore, direct counting of TCA precipitates could be used to measure the amount of choline-^{14}C in phosphatidyl choline or the amount of glycerol-^{3}H in phospholipids plus neutral glycerides.

Characteristics of Incorporation of Radioactive Precursors into Cellular Lipids

Incorporation of choline-^{14}C into the acid-insoluble fraction of cells reached its maximum rate about 45 min after introduction of radioactive choline into the medium (Fig. 1 A, one of three identical experiments). Coinciding with the beginning of rapid incorporation of labeled choline into phosphatidyl choline (acid-insoluble fraction), the rate of increase in specific activity of the soluble intracellular pool slowed down, but did not level off. Thus, a large and increasing intracellular pool of soluble radioactivity was maintained throughout incorporation.

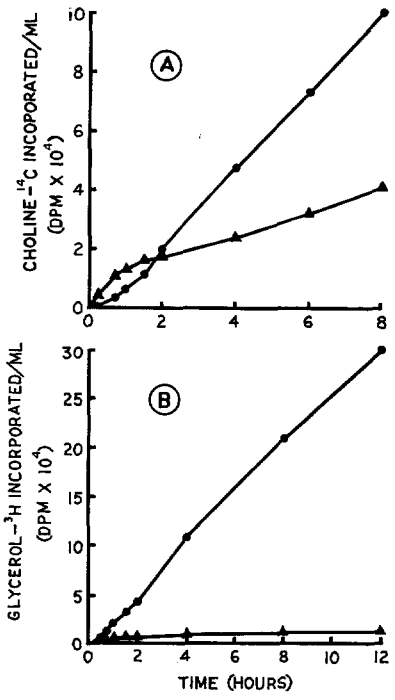

FIGURE 1 Incorporation of choline-^{14}C (Fig. 1 A) and glycerol-^{3}H (Fig. 1 B) into cells. Exponentially growing cells were suspended at a concentration of $\sim 10^6$ cells per milliliter of medium with 1 μCi choline-^{14}C/ml (132 nmoles/ml) (1 A) or 5 μCi glycerol-^{3}H/ml (10 nmoles/ml). At intervals after introduction of radioisotopes, duplicate 1 ml samples of well-mixed cell suspensions were removed; the cells were harvested and analyzed for acid-soluble (▲——▲) and acid-insoluble (●——●) radioactivity. Each point in Figs. 1–6 represents the average of duplicate samples.

In contrast to choline-^{14}C, incorporation of glycerol-^{3}H into phospholipids and neutral glycerides (acid-insoluble fraction) was evident immediately after exposure of the cells to the radioactive label (Fig. 1 B, one of two identical experiments). Furthermore, no appreciable soluble pool of radioactive precursors accumulated, demonstrating that essentially all of the glycerol-^{3}H which entered the cells was quickly incorporated into lipids.

Characteristics of Turnover of Radioactive Labels in Cellular Lipids

When different concentrations of nonradioactive choline (0.033, 3.3, and 16.5 μmole/ml) were added to the fresh medium of three identical, labeled cell cultures, the measurable rates of turnover of the choline-^{14}C in phosphatidyl choline (acid-insoluble fraction) increased with increasing concentrations of nonradioactive choline (Fig. 2, one of three identical experiments). The highest concentration (16.5 μmoles nonradioactive choline/ml medium) inhibited growth and induced encystation. However, no effect on growth was observed with the lower concentrations (i.e., 0.33 and 3.3 μmoles/ml). The soluble intracellular pool of radioactive molecules did not increase enough to account for the loss of radioactivity from phosphatidyl choline (acid-insoluble fraction) in any of the turnover experiments (see Fig. 2); therefore, radioactivity must have been lost from the soluble cell pool to the medium.

Indication that this was the case was obtained when labeled cells were grown in medium supplemented with 3.3 μmoles nonradioactive choline/ml. Then the loss of radioactivity to the medium was measurable for $\sim$70 hr, after which an equilibrium was reached between radioactivity in the cells and that in the medium (Fig. 3, one of two identical experiments). At each of the time points shown in Fig. 3, radioactive medium was cleared of cells by low-speed centrifugation. Scintillation counting of the acid-insoluble fractions of the medium revealed no radioactivity. Therefore, the molecules that escaped into the medium probably were free choline-^{14}C, unless a soluble form of phosphorylated choline, such as phosphorylcholine or cytidinediphosphatecholine, had passed into the medium.

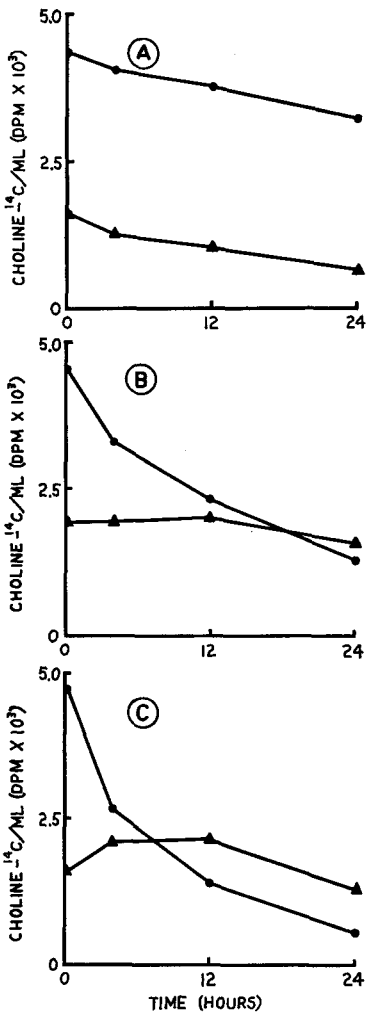

FIGURE 2 Turnover of choline-^{14}C in the presence of nonradioactive choline in the growth medium. Exponentially growing cells were suspended at a concentration of $\sim 10^6$ cells per milliliter in fresh medium supplemented with 0.25 μCi choline-^{14}C/ml (0.033 μmoles/ml). After 12 hr (0 hr on graph), the cells were washed and resuspended at a concentration of $\sim 10^6$ cells per milliliter in fresh medium supplemented with 0.033 (Fig. 2 A), 3.3 (Fig. 2 B), or 16.5 (Fig. 2 C) μmoles nonradioactive choline/ml. The cultures were analyzed as in Fig. 1. Symbols are given in Fig. 1.

In summary, addition of nonradioactive choline to the medium caused a loss of radioactivity from phosphatidyl choline into the soluble cell pool, which, in turn, equilibrated with the medium. Apparently, nonradioactive choline either induced an increased turnover of the entire phosphatidyl choline molecule or caused an "exchange" of the labeled choline moiety of phosphatidyl choline.

To distinguish between these two alternative explanations, the characteristics of glycerol-^{3}H–labeled phospholipids were examined. No turnover of glycerol-^{3}H–labeled, acid-insoluble material (total cellular lipids) was apparent, and essentially no acid-soluble pool of radioactive molecules could be detected in cells chased with 3.3 μmoles nonradioactive choline/ml (Fig. 4, one of three identical experiments). The results were identical if the medium had been supplemented

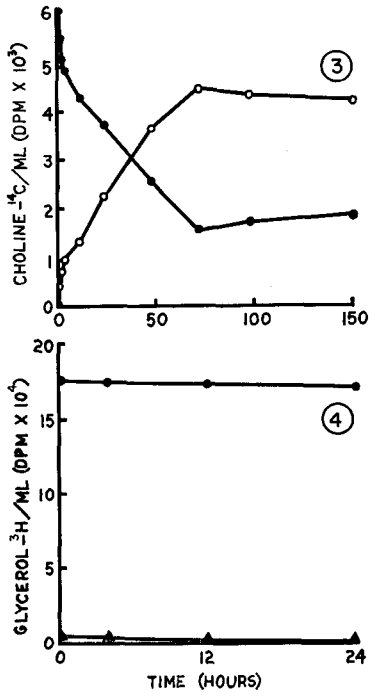

FIGURE 3 Loss of cellular choline-^{14}C to the medium. Exponentially growing cells were labeled as described in Fig. 2 and chased, beginning at time 0, with 3.3 μmoles nonradioactive choline/ml medium. ●——●, radioactivity of cells; ○——○, radioactivity of medium.

FIGURE 4 Turnover of glycerol-^{3}H in the presence of 3.3 μmoles nonradioactive choline/ml medium. Exponentially growing cells were labeled in medium containing 2 μCi glycerol-^{3}H/ml (4 nmoles/ml) and chased, beginning at time 0, with 3.3 μmoles nonradioactive choline/ml medium in a manner similar to that described in Fig. 2. Identical results were obtained if the chase was 0.033 or 16.5 μmoles nonradioactive choline/ml, or 16.5 μmoles nonradioactive glycerol/ml, with the exception that the cells did not grow in medium containing 16.5 μmoles nonradioactive choline/ml. Symbols are given in Fig. 1.

TABLE I

Turnover of Glycerol-^{3}H–Labeled Phospholipids in the Presence of Large Concentrations of Nonradioactive Choline or Glycerol

Concentration of chase	Experiment No.	Time in chase medium: 0 hr	Time in chase medium: 24 hr
		dpm/μmole lipid P	*dpm/μmole lipid P*
16.5 μmoles glycerol/ml	1	508,171	246,500 (456,000)*
	2	406,895	182,140 (355,085)*
16.5 μmoles choline/ml	1	460,018	451,000 (451,000)*
	2	628,030	584,830 (584,830)*

Cells from four exponentially growing cultures were suspended at a concentration of $\sim 10^6$ cells per milliliter in fresh medium containing 2 μCi glycerol-^{3}H/ml (4 nmoles/ml). After a 12 hr labeling period, the cells were washed and resuspended to a concentration of $\sim 10^6$ cells per milliliter in fresh medium containing the indicated concentrations of nonradioactive glycerol or choline. Duplicate samples were taken immediately (0 hr) or 24 hr later, and analyzed for the radioactivity in phospholipids.

* The specific activities corrected for the observed growth dilution are given in parenthesis. No growth was observed when 16.5 μmoles choline/ml was added to the medium, whereas growth was normal (i.e., 27 hr doubling time) when 16.5 μmoles glycerol/ml was used.

with 0.033 or 16.5 μmole nonradioactive choline/ml or 16.5 μmole nonradioactive glycerol/ml (not illustrated). On the other hand, glycerol-^{3}H in the phospholipid fraction of the total lipids did turn over at a slow rate which was not accelerated by the addition of large concentrations of either nonradioactive glycerol or choline (Table I).

Inasmuch as no turnover of glycerol-^{3}H was apparent in the total cellular lipids (Fig. 4), although some turnover was observed in isolated phospholipids (Table I), it seemed likely that some of the glycerol-^{3}H in phospholipids had been converted to neutral lipid. To test this possibility, the percentage of total lipid radioactivity in neutral lipids was analyzed in cells grown for 12 hr in medium containing 2 μCi glycerol-^{3}H/ml (4 nmoles/ml) as well as in cells subsequently cultured for 24 hr in chase medium containing 16.5 μmoles nonradioactive glycerol/ml. In two identical experiments the radioactivity of total cellular neutral lipids increased from about 33% during incorporation to ~38% after 24 hr in medium containing nonradioactive glycerol.

Cell-Free Incorporation and Turnover

In order to determine whether an "exchange" reaction was operational in vitro, cell-free mixtures of microsomal membranes were incubated with choline-^{14}C. Incorporation of choline-^{14}C into the acid-insoluble fraction of the microsomes increased as the protein concentration of the fraction was increased (Fig. 5, one of three identical experiments). Chromatography demonstrated that all of the acid-insoluble radioactivity was in phosphatidyl choline. A divalent cation requirement was suggested by the inhibition of incorporation in the presence of ethylenediaminetetraacetic acid, (EDTA) (Fig. 5). Glycerol-^{3}H was not incorporated by this cell-free system when substituted for choline-^{14}C in the reaction mixture. As much as 132 μmoles of nonradioactive choline, added to the reaction mixture along with the radioactive glycerol, did not induce any measurable exchange of glycerol. Thus, as with living cells, only the choline moiety of phosphatidyl choline seemed to be involved in this phenomenon.

Net incorporation of choline-^{14}C into phosphatidyl choline by cell-free microsomes was initially linear as a function of time, but stopped after 30–45 min (Fig. 6, one of two identical experiments). The addition of concentrated nonradioactive choline, either before or after this leveling off, not only stopped measurable incorporation of choline-^{14}C but effected a rapid turnover of label in the microsomal phosphatidyl choline as if free and bound choline were in equilibrium.

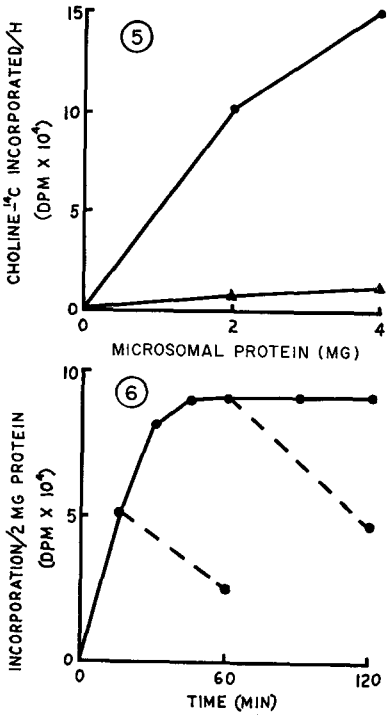

FIGURE 5 Cell-free incorporation of choline-^{14}C into microsomal phosphatidyl choline as a function of the protein concentration. Cell-free reaction mixtures containing 2 or 4 mg microsomal protein were incubated with 1 μCi choline-^{14}C (132 nmoles/ml) for 60 min of incorporation at 29°C, when the reactions were terminated by the addition of 10% (final concentration) TCA. ●——●, microsomes; ▲——▲, microsomes + 0.05 μmoles disodium EDTA.

FIGURE 6 Incorporation of choline-^{14}C into cell-free microsomal suspensions and the effect of chasing with nonradioactive choline. 2-mg samples of microsomal protein were incubated as for Fig. 5. In the reactions terminated at 60 and 120 min, nonradioactive choline (●----●) or buffer alone (●——●) had been added at 15 and 60 min, respectively. (●----●: 132 μmoles nonradioactive choline in 0.1 ml of 0.1 M phosphate buffer [pH 7.4]; ●——●: 0.1 ml of 0.1 M phosphate buffer [pH 7.4].)

Intracellular Localization of Labeled Glycerides

Because glycerol-^{3}H was more stable as a phospholipid label than choline-^{14}C, it was important to determine its utility as a marker of membrane phospholipids. To this end, the percentages of radioactivity incorporated into phospholipids and neutral lipids of microsomes and of lipid droplets isolated from labeled cells were compared.

Phospholipids comprised ~96% of the radioactive lipids in microsomes but only ~2% of that in lipid droplets (Table II). The ratio of total radioactivity in microsomes and lipid droplets (70%/30%) was similar to the distribution of glycerol-^{3}H in total cellular lipids (phospholipids 67%/neutral lipids 33%), as described above. Therefore, although the quantitative recoveries of radioactivity achieved in fractionations are not known, it would appear that most of the phospholipids in the cell are confined to membranes, whereas essentially all of the neutral lipid is in lipid droplets.

DISCUSSION

As observed by Nagley and Hallinan (2) in rat liver cell fractions and by Plagemann (4, 5) in rat hepatoma cells, essentially all of the acid-insoluble choline-^{14}C was localized in the phosphatidyl choline of growing *A. palestinensis*.

In the ameba, the choline moiety of phosphatidyl choline was assumed to be in equilibrium with free choline in the cell and in the extracellular medium (Figs. 2 and 3). The addition of large concentrations of nonradioactive choline induced a turnover of the radioactivity already incorporated into phosphatidyl choline.

In contrast, the apparent rate of turnover of phospholipids labeled with glycerol-^{3}H was slow and was not affected by the addition of nonradioactive choline or glycerol (Table I). Phosphatidyl choline has been reported to comprise 45% of the phospholipids in a similar soil ameba (28); thus, any change in its rate of turnover should have been reflected in measurements of the total phospholipid fraction. Therefore, it seems likely that the observed turnover of choline-^{14}C involved only the choline moiety of phosphatidyl choline and not the entire phospholipid molecule. In other words, the phenomenon probably was due to an exchange reaction rather than to the degradation and *de novo* synthesis of phosphatidyl choline.

Neither the soluble form of choline involved nor the mechanism of exchange in these cells is known. In growing rat hepatoma cells, where the possibility of a choline exchange reaction has been suggested by Plagemann (5), only phosphorylcholine was found in the soluble cell pool. On the other hand, cytidinediphosphatecholine has been reported to be present and in equilibrium with

TABLE II

Distribution of Glycerol-^3H in Neutral Lipids and Phospholipids of Microsomes and Lipid Droplets

Cell fraction	Experiment No.	dpm/10^6 cells		% in phospholipids	Relative total radioactivity in fraction*
		Neutral lipids	Phospholipids		
					%
Microsomes	1	298	6437	96	68
	2	114	2514	96	72
Lipid droplets	1	3118	43	2	32
	2	1007	24	2	28

Exponentially growing cells were labeled for 12 hr with 2 μCi glycerol-^3H/ml medium (4 nmoles/ml) as described in Table I.

* (Radioactivity in neutral lipids + phospholipids of fraction)/ (radioactivity in microsomes + lipid droplets). × 100.

phosphatidyl choline in both normal (29) and choline-deficient (17) rat liver. In choline-deficient liver, an increased synthesis and secretion of plasma lecithin and not an exchange of choline reportedly results from the administration of large doses of choline (7, 30). However, the latter results have been challenged by Treble et al. (18) who presented evidence that a base exchange of choline to phosphatidyl choline occurs in vivo in liver at four times the rate at which phosphoryl choline is converted to lecithin.

The rapid attainment of an equilibrium between free choline-^{14}C and the radioactive moiety of phosphatidyl choline in cell-free microsomes (Fig. 6) was in sharp contrast to the slower kinetics of this reaction in living cells (Figs. 2 and 3) which perhaps was limited by a slow rate of transfer of choline in and out of the cells. Consistent with this, Plagemann and Roth (31) have reported that transport is the rate-limiting step in the incorporation of choline into the acid-insoluble pool of rat hepatoma cells in vitro. The enzyme(s) or cofactor(s) needed for the exchange reaction must have been bound to membranes, since only inorganic ions and choline were added to the microsomal fraction. Cell-free exchange reactions, similar to the phenomenon described here, have been reported for choline (10), serine (11, 12), ethanolamine (13, 14, 15), and inositol (16).

Under normal growth conditions, no exogenous choline is required by these cells (21). However, cell growth and morphology appeared normal in medium supplemented with up to 3.3 μmoles nonradioactive choline/ml, a concentration at which the choline-^{14}C moiety of phosphatidyl choline was assumed to be exchanging (Fig. 2 B). While it is hard to envision exchange reactions in equilibrium with the natural environment as being biologically significant to soil ameba, the observations of this study could represent the amplification of a local and normally occurring intracellular process. Inasmuch as phospholipids are important components of cellular membranes, it is conceivable that exchange reactions play some role in membrane function.

The implication of these experiments on the use of choline-^{14}C as a marker for membrane phosphatidyl choline in *A. palestinensis* is obvious; it is an unsuitable label if large chase concentrations of nonradioactive choline are used, because of the resulting instability of the label.

In contrast, glycerol-^3H was found to be a relatively stable marker of cellular phospholipids. It also appears to be a specific marker for membrane phospholipids. Although ~33% of the incorporated glycerol-^3H was recovered from cellular neutral lipids, most of these neutral lipids were confined to lipid droplets, whereas the glycerol-^3H in membranes was almost entirely phospholipid (Table II).

The apparent rate of turnover of total cellular phospholipids labeled with glycerol-^3H was not affected by large chase concentrations of nonradioactive choline or glycerol (Table I). During the course of turnover the amount of label in cellular neutral lipids relative to cellular phospholipids increased slightly. Because little, if any, turnover of total cellular lipids (acid-insoluble material)

was apparent (Fig. 4), it seems likely that some membrane phospholipids were slowly converted to neutral lipids and transferred to lipid droplets.

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