Complete Retention of Phospholipid Acyl Groups by Mammalian Cells in Culture†

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ABSTRACT: Radiolabeled phosphate, acetate, and glycerol are incorporated into strain L-fibroblast phospholipids. The acetate and glycerol specifically label the fatty acid and glycerol moieties, respectively, of the phospholipids. To study the metabolic fate of the various moieties of phospholipids, cells incubated with the above radiolabeled compounds were transferred to unlabeled medium, and the rate at which phospholipid radioactivity per 10^6 cells decreased was determined. The rate of decrease expected on the basis of cell division alone was estimated either by monitoring increases in cell number, or by measuring the rate at which radiolabeled DNA per 10^6 cells decreased. Both phospholipid phosphorus and glycerol are lost at a rate greater than can be accounted for by cell division alone. By contrast, nearly all phospholipid acyl chains were retained by the cell to the same extent as radiolabeled DNA. While presence of nonradioactive glycerol in the medium increased the rate at which glycerol was lost from phospholipid, the addition of exogenous fatty acid was without effect on the retention of phospholipid acyl groups. The acyl–glycerol bond of phosphatidylcholine is metabolically more labile than that of phosphatidylethanolamine. Together the data suggest that although L-fibroblast phospholipids undergo deacylation–reacylation reactions, the acyl chains do not equilibrate with either extracellular or intracellular pools of unesterified fatty acid.

Studies with mammalian tissues have shown that there are at least two principal mechanisms by which fatty acids are incorporated into the phospholipids of cell membranes. One entails the synthesis de novo of phospholipid molecules and results in a net increase of phospholipid (Kennedy and Weiss, 1956). The other involves exchange reactions in which there is no concomitant increase in the amount of phospholipid (Lands, 1960; Reed, 1968). Two types of exchange reaction have been described. The first has been demonstrated in erythrocytes (Reed, 1968), and involves the exchange of entire intact membrane phospholipid molecules with phospholipid present in the medium. A second more conservative type has been reported in erythrocytes (Shohet et al., 1968), brain (Dhopeshwarkar et al., 1971), liver (Holub et al., 1971; Krause and Beamer, 1972), lymphocytes (Resch et al., 1972; Ferber and Resch, 1973), and lung (Tierney et al., 1967), and depends on an acyl transferase catalyzed exchange between acyl CoA and membrane lysophosphoglycerides (Lands, 1960; Hill and Lands, 1970). As a consequence of such exchange reactions the cell has a mechanism by which membrane phosphoglyceride fatty acid composition may be modified without a net increase in membrane phospholipid. It is likely that in some cells both exchange mechanisms are responsible for the turnover of phospholipid fatty acids. The biological significance of this turnover has been demonstrated in experiments with whole animals in which the phospholipid fatty acid composition of tissues was shown to be modified by dietary manipulations (Allman et al., 1965). Turnover of liver membrane phospholipid labeled with [^{14}C]acetate occurs with a $T^{1/2}$ of approximately 2 days (Lee et al., 1973).

Turnover of mammalian cell phospholipid has also been studied in cells cultured in vitro. Using mast cells, Pasternak and Bergeron (1970) demonstrated turnover of the nonacyl moieties of phospholipids. It was not possible to determine from their data to what extent the phospholipid acyl groups underwent turnover. In another study (Peterson and Rubin, 1969) chick embryo fibroblasts, prelabeled with choline, were found to release radioactivity into the medium in lipid soluble form, suggesting the loss, possibly by exchange, of intact phospholipid molecules.

During the adaptation of monolayer cultures of strain L-fibroblasts to a serum and lipid-free medium, Geyer et al. (1962) reported a decrease in the polyunsaturated fatty acid content of cellular phospholipid. The rate of decrease correlated directly with the rate of cell division and suggested essentially complete conservation of these acids. It was also shown that radioactivity incorporated into cellular phospholipids during incubation of strain L-fibroblasts with [2-^{14}C]acetate was completely retained in the phospholipid fraction over a period of 8–10 generations after transfer of the labeled cells to unlabeled medium (Geyer, 1967). This was also reflected by the absence of $^{14}CO_2$ formation during the same period. The results were unaffected by the presence or absence of serum protein and lipids in the culture medium. Warren and Glick (1968), using radiolabeled glucose as a source of lipid precursor, showed that the lipid of the outer membrane of strain L-fibroblasts in the log phase of growth was lost from the cell at a rate slightly greater than can be accounted for by cell multiplication. The discrepancy between these data and those of Geyer (1967) may have arisen in part from differences in the precursor used to label the phospholipid.

In the current study the relative and absolute rates of loss of various moieties of the phospholipids of strain L-fibroblasts were determined after labeling with specific radioactive precursors. The following data will show that, in con-

† From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115. *Received June 22, 1975.* This work was supported by the Department of Nutrition Fund for Research and Teaching and U.S. Public Health Service Research Grant 1 RO1 HL-15646-01A1.

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trast to the rest of the phospholipid molecule, a large fraction of the phospholipid acyl groups is completely conserved in the phospholipid fraction.

Materials and Methods

Materials. Radioactive compounds: [2-^{3}H]glycerol, 200 Ci/mol; [2-^{3}H]acetate, 200 Ci/mol; [2-^{14}C]acetate, 50 Ci/mol; [*methyl*-^{14}C]thymidine, 50 Ci/mol; and [^{32}P]phosphate, 100 Ci/mol, were purchased along with Aquasol and Hyamine from New England Nuclear Corporation. Aqueous solutions of the radiolabeled compounds were sterilized by passage through 0.22-μ Millipore filters before use. Lipid standards and oleic and linoleic acids from Applied Science Lab., Inc., were dissolved in redistilled heptane and stored at −24°C. Cell culture medium, MB 752/1, associated Biomedic Systems, Inc., was used as a suspension medium by dissolving the powder in deionized distilled water, and adding the following components to give the indicated final concentrations: Methocel, 15 Hz, Dow Chemical Co., 0.025%; $CaCl_2$, 19.6 mg/l.; and thymidine, 30 μM. Horse serum, Grand Island Biological Co., used to supplement the culture medium was delipidized by an ethanol–diethyl ether extraction procedure (Albutt, 1966) prior to use. The method, modified to accommodate 100-ml batches of fourfold concentrated serum, was found to remove 94–96% of the esterified and unesterified fatty acid. Phospholipase C (Type I) from *C. welchii* was purchased from Sigma Chemical Co.

Tissue Culture. Mouse strain L-fibroblasts, serially transferred in this laboratory for 15 years, were cultured at 37 ± 0.3°C in 125-ml screw cap Erlenmeyer flasks containing 50 ml of suspension medium. The pH was 7.2 and osmolarity, as determined using an Osmette, Precision Systems, was 295–300 mOs/l. An Incubator Shaker Model G-27, New Brunswick Scientific Co., Inc., was used to agitate the culture at 120 rpm. Stock cultures were antibiotic-free, but penicillin, 10000 units, and streptomycin, 0.01 g, were added to experimental cultures as a precaution when frequent rinses or transfers were involved. Under the culture conditions described, cells will grow from 5.0×10^4/ml to 1×10^6/ml, without a medium change, and with a mean generation time of approximately 24 hr. Cell numbers and sizes were obtained with a Coulter counter and particle size distribution plotter. Log phase was maintained by keeping the cell density between 3×10^5 and 1×10^6 cells/ml.

Radiolabeling of Cellular Lipids and DNA. Cultures at a density of $2.5–5.0 \times 10^5$ cells/ml were incubated for 24–48 hr in the presence of appropriate radiolabeled precursors. When [*methyl*-^{14}C]thymidine was used, the thymidine supplement was omitted from the culture medium. At the end of the isotope incorporation period the cells were centrifuged at 600*g* for 5 min at room temperature, and then washed twice by resuspending in 25 ml of fresh, warmed, unlabeled medium, followed by recentrifugation. Some of these cells were immediately harvested and analyzed to obtain zero-time data. The remainder were resuspended in fresh unlabeled medium and further incubated to study the subsequent depletion of isotope. At selected intervals during the depletion period aliquots of the culture, containing between 10 and 30×10^6 cells, were removed, washed twice with 0.9% NaCl containing 0.025% methyl cellulose, and assayed for lipid soluble radioactivity. To minimize problems associated with recycling of label during the depletion period, the medium was changed daily for the first 2 days, and then every other day until the last (8th) day. In those experiments in which the rate of decrease of cellular [*methyl*-^{14}C]thymidine DNA content was compared with that of ^{3}H-labeled phospholipid a second aliquot containing $2–9 \times 10^6$ cells was taken from the same culture, washed twice with saline, and dissolved directly in Hyamine for determination of ^{14}C content.

Extraction and Isolation of Lipids. After washing, the cells harvested for lipid extraction were mixed with 2.5 ml of methanol, and then 5.0 ml of $CHCl_3$ was added. Details of the extraction are published elsewhere (Marinetti et al., 1958). Briefly, this involves three extractions with 2:1 (v/v) chloroform–methanol and washing as described by Folch et al. (1957). By this means at least 98% of the cellular radioactivity was extracted in lipid-soluble form. After adjusting to a known volume, an aliquot of extract was assayed for total lipid radioactivity. The remaining extract was concentrated under N_2 and transferred to a thin-layer plate coated (0.3 mm) with silica gel H impregnated with 1% ammonium sulfate (Kaulen, 1972). These plates were routinely activated for 30 min at 100°C just prior to use. The neutral lipids were separated into their subclasses in solvent system I, which is a mixture of hexane–diethyl ether–acetic acid, 60:30:1 (v/v). In solvent system I all the phospholipids remained at the origin. Lipid fractions were identified after staining with iodine vapors by comparison of the sample R_f's with those of standard chromatographed under the same conditions.

Separation of the various phospholipid classes was effected by first spotting the lipid extracts on thin-layer plates and developing the plates in solvent system I. After drying under N_2 for 5 min, the chromatogram was developed again in the same direction, but in solvent system II, composed of chloroform, methanol, acetic acid, and water, 50:30:8:4 (v/v) (Skipski et al., 1963). The solvent was allowed to migrate to the top of the plate. Sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were well resolved by this procedure; however, because the separation between phosphatidylserine and phosphatidylinositol was not always satisfactory, radioactivity data from these fractions were combined. After development the individual lipid classes were visualized by briefly exposing the plate to iodine vapors and the appropriate gel areas were scraped into liquid scintillation vials.

Radioactivity Determination. The intact cell pellet was dissolved in Hyamine. Ten milliliters of Aquasol was added, followed by enough water to cause the formation of a stiff translucent gel. To eliminate chemiluminescence, the pH of the mixture was adjusted to 4.5 with a small amount of glacial acetic acid before counting. Total lipid and neutral lipid radioactivity were determined after dissolving in 2:1 (v/v) toluene–ethanol solution containing 0.4% omnifluor. Samples containing phospholipid adsorbed to silica gel were mixed with 1 ml of water before the addition of Aquasol (Webb and Mettrick, 1972).

All radioactivity measurements were made with a Beckman Model LS-30 liquid scintillation system equipped for the simultaneous assay of both ^{3}H- and ^{14}C-labeled compounds. Corrections for quenching were made by using an external standard.

When the total phospholipid fatty acid composition was to be measured, care was taken to remove the sample from the plate immediately after development of the chromatogram in solvent system I, and prior to exposure to I_2 vapors. The origin, containing the phospholipid, was scraped into a small screw cap tube containing 1 ml of 1.0 *N* NaOH in a

60:40 (v/v) mixture of methanol and benzene (Glass, 1971). After reacting for 5 min at room temperature, the fatty acid methyl esters were extracted from the mixture into 2 ml of heptane. This solvent was then transferred to a small test tube, where it was removed under a stream of N_2 and the sample immediately redissolved in 5–10 μl of heptane. Separation and quantitation of methyl esters were accomplished by injecting 0.5 μl of the heptane solution onto a 6-ft glass column which was packed with 10% SP-222-PS (Supelco, Inc.) and mounted in an F&M gas chromatograph equipped with a flame ionization detector, and electronic integrator (Hewlett Packard Model 3370B). Identification of sample peaks was made by comparison of their retention times with those of known standards.

Saponification Procedure. Lipid from approximately 40 $\times$ 10^6 cells which had been preincubated with the appropriate radiolabeled precursor was dissolved in 1.4 ml of 0.11 *N* NaOH in chloroform–methanol, 2:1 (v/v). Ten minutes later, 0.2 ml of methanol and 0.5 ml of 1.0 *N* aqueous HCl was added. After vortex mixing the upper phase was transferred to a liquid scintillation vial. The lower phase was reextracted twice more with 0.8 ml of distilled water (Hajra et al., 1968). These water washes were combined with the original upper phase; 10 ml of Aquasol was added and the radioactivity determined. The chloroform phase was dried; the residue was redissolved in 2–3 drops of chloroform–methanol, 1:1 (v/v), and then transferred to a thin-layer plate which was developed in solvent system I. After development, the radioactivity of the various lipid fractions was assayed.

Hydrolysis of Phospholipid by Phospholipase C from C. welchii. Radiolabeled lipid from 30 $\times$ 10^6 strain L-fibroblasts was dissolved in 1.0 ml of diethyl ether, in a small screw cap test tube. To this was added one unit of phospholipase C dissolved in 0.25 ml of 50 m*M* potassium phosphate buffer (pH 7.0) containing 1% albumin (Mavis et al., 1972). The two phases were mixed vigorously for 5 min on a vortex mixer. Phase separation occurred rapidly when agitation was stopped. The ether phase was removed and evaporated to dryness; the residue was dissolved in a few drops of chloroform–methanol (1:1) for transfer to a thin-layer plate for separation of neutral lipids in solvent system I. Radioactivity of the various fractions was measured as described above. After thorough ether washing the water phase was quantitatively transferred to a scintillation vial, and the radioactivity measured after the addition of Aquasol.

Results

Strain L-fibroblasts incubated with either [2-^{14}C]acetate or [^{32}P]phosphate, incorporated appreciable label into their phospholipids. When transferred to fresh unlabeled medium, and sampled at various intervals up to 72 hr, a decline in radioactivity of the cellular phospholipids was observed with both isotopes on a per cell basis. (Care was taken to change the medium daily to prevent recycling of label.) However, if account was taken of the decrease of radioactivity per cell resulting from cell division alone, a marked difference between the retention of the ^{14}C and ^{32}P was obvious when the data were plotted (Figure 1). Had the cells retained all of any given radioactive phospholipid precursor except that which was lost by dilution due to cell division, a line parallel to the time axis would have resulted. This was the case for phospholipid labeled with [2-^{14}C]acetate but not with [^{32}P]phosphate. Despite the fact that 70% of the

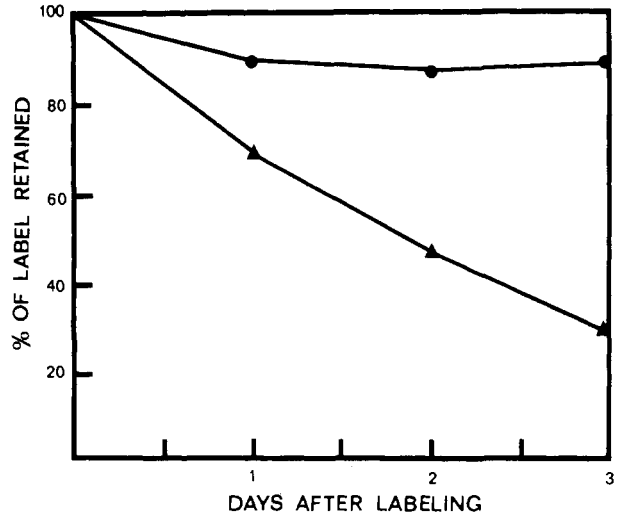

FIGURE 1: Depletion of radiolabel from phospholipids of cells preincubated with either [^{32}P]phosphate or [2-^{14}C]acetate. Cells incubated with 10 μCi of [2-^{14}C]acetate or 50 μCi of [^{32}P]phosphate for 24 hr were transferred to nonradioactive medium for an additional 72 hr. At the times shown, the phospholipid radioactivity per 10^6 cells was measured. Using cell counts the data were mathematically corrected for changes in radioisotope content resulting solely from cell multiplication. Data on the ordinate represent the fraction of radioactivity remaining in the phospholipid; the zero time value was taken as unity. Results are the mean of three experiments. (●) Phospholipid from cells incubated with [2-^{14}C]acetate; (▲) phospholipid from cells incubated with [^{32}P]phosphate.

[^{32}P]phosphate originally incorporated into phospholipid was lost during the 72 hr following removal of cells from their radioactive medium, 90% of the [2-^{14}C]acetate originally incorporated was retained. Nearly all of the 10% loss observed in the latter case occurred during the first 24 hr.

To better assess the retention or loss of the various moieties of the cellular phospholipids over longer periods of time, cells in most of the remaining experiments were incubated simultaneously with [*methyl*-^{14}C]thymidine and a radioactive ^{3}H-labeled phospholipid precursor. The rate of decrease of ^{14}C-radioactivity/10^6 cells, which was attributable to cell multiplication only, was then directly compared with the rate of decrease of ^{3}H-labeled phospholipid radioactivity/10^6 cells. Results from such an experiment are shown in Figure 2. There was little change in the ^{14}C-content per cell during the first 24 hr indicating that little or no cell multiplication or death had occurred during this time. This lag in growth after several centrifugations is not unusual in these cells. From the second through the eighth day, however, the ^{14}C-radioactivity per 10^6 cells decreased logarithmically with a slope, $b = -0.2684$ (Table I). The b value calculated on the basis of a 24-hr doubling time is -0.3010. Since the results shown in Table I were from experiments in which the cell doubling time, based on cell numbers, was between 24 and 28 hr, b values between -0.2599 and -0.3010 were expected. The rate at which the ^{3}H-labeled phospholipid content/10^6 cells decreased during the first 24 hr was greater than that of the [^{14}C]DNA content/10^6 cells. From the first to the eighth day, however, the rates of decrease of both the H-labeled phospholipid, $b = -0.2528$, and [^{14}C]DNA, $b = -0.2684$, were not statistically different. Because of the variations occurring during the first day after the labeling period, the zero-time value was omitted from slope calculations. Throughout the period from the first through the eighth day, while the cell mass

Table I: Depletion Rates of Radiolabeled Phospholipid and DNA from Strain L-Fibroblasts.[a]

Sources of DNA or Phospholipid Radioactivity			Fatty Acid Present during Depletion[b]	Slope of Regression Line from Days 1–8 of Depletion[c]	
[CH_3-^{14}C]-Thymidine	[2-^{3}H]-Acetate	[2-^{3}H]-Glycerol		DNA	Phospholipid
+	+	–	–	−0.268 ± 0.056	−0.253 ± 0.080
+	+	–	+	−0.289 ± 0.053	−0.286 ± 0.057
+	–	+	–	−0.282 ± 0.070	−0.343 ± 0.052

[a] Data from days 1–8 of experiments described in Figures 2, 3B, and 4 were used to calculate the rate of decrease of radioactivity per 10^6 cells from the phospholipid and DNA of strain L-fibroblasts. The rate is calculated as the slope (b) of a nonlinear regression line, using the formula: $b = (n\Sigma \log y - (\Sigma x)(\Sigma \log y))/[n\Sigma x^2 - (\Sigma x)^2]$; where n = 15 (three determinations at five points each), x = days after transfer to nonradioactive medium, and y = picomoles or radiolabeled precursor retained per 10^6 cells in either the phospholipid or DNA fractions. [b] Where indicated an equimolar mixture (8.8 μM) of oleic and linoleic acids was added to the nonradioactive depletion medium. [c] The significance of the difference between the slopes of the DNA and phospholipid regression lines was estimated using Student's t test. A statistically significant difference between the slopes of regression lines for the decrease in radioactivity in DNA and phospholipid fractions was found only when the phospholipid glycerol was radiolabeled ($p << 0.001$).

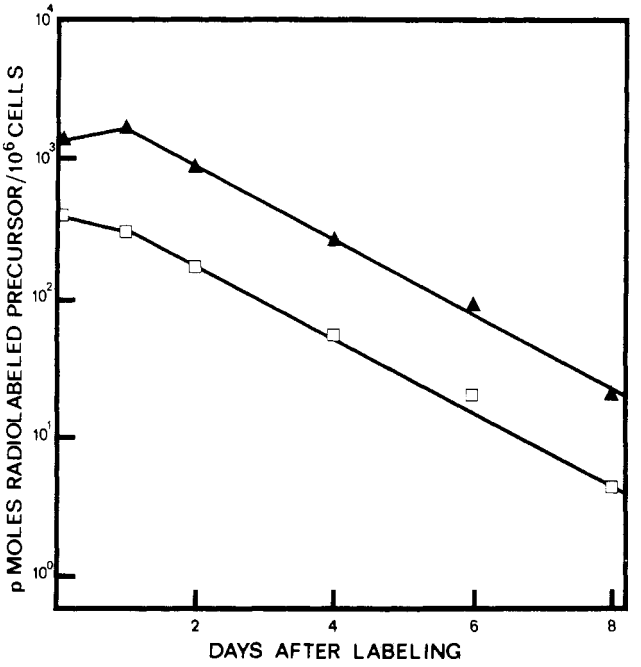

FIGURE 2: Retention of radiolabeled phospholipid acyl groups and DNA by L cells. Cells were incubated for 48 hr with a mixture of 5 μCi of [*methyl*-^{14}C]thymidine and 50 μCi of [2-^{3}H]acetate. At intervals after transfer to nonradioactive medium, the [^{14}C]DNA and ^{3}H-labeled phospholipid content per 10^6 cells was assayed. Each point is the average of three determinations. See Table I for statistical evaluation of the slopes of the regression lines calculated from the data obtained on days 1–8. (▲) [*methyl*-^{14}C]Thymidine-labeled DNA; (□) ^{3}H-labeled phospholipid acyl groups. For details of procedures see Materials and Methods.

Table II: Percent Distribution of Radioactivity in Lipids of Strain L-Fibroblasts Incubated with [2-^{3}H]Acetate or [2-^{3}H]Glycerol.[a]

Radioactive Precursor	Relative ^{3}H Distribution[b]		
	Phospholipid (%)	Sterol (%)	Others (%)
[2-^{3}H]Acetate	67.6 ± 1.90	30.1 ± 2.10	2.3 ± 0.20
[2-^{3}H]Glycerol	94.7 ± 0.12	3.4 ± 0.10	1.9 ± 0.06

[a] Cells were incubated for 48 hr with 50 μCi of either [2-^{3}H]-acetate or [2-^{3}H]-glycerol. They were then harvested and the distribution of label between the lipid classes was determined. For details of lipid extraction, thin-layer chromatography and radioassay techniques see Methods and Materials. [b] Results are expressed as percent of total lipid radioactivity in a given lipid fraction and are presented as the mean ±SD of four experiments.

increased by a factor of approximately 65, the radioactivity incorporated into phospholipid from [2-^{3}H]acetate was as metabolically stable as the [*methyl*-^{14}C]thymidine incorporated into DNA, the rate of decrease of both isotopes being equal to each other and to that predicted on the basis of dilution resulting from cell division alone. Analysis of cellular lipid revealed that the [2-^{3}H]acetate was incorporated principally into the phosphoglyceride and sterol fractions (Table II). Of the radioactivity in the phosphoglyceride fraction, 99.5% was recovered either as fatty acid or fatty acid methyl ester after alkaline methanolysis (Table III). By contrast, incubating the cells with [2-^{3}H]glycerol resulted in the biosynthesis of phosphoglycerides specifically labeled in the glycerol moiety as shown by the following results. (1) Nearly all of the radioactivity was incorporated into the phosphoglyceride fraction (Table II) and was rendered water soluble after alkaline methanolysis (Table III). (2) None of the phosphoglyceride radioactivity of cells incubated with [2-^{3}H]glycerol was present in the ethanolamine or choline fractions, since incubation of the isolated phosphoglycerides with phospholipase C from *C. welchii* resulted in the formation of only negligible quantities of water-soluble radioactivity (Table III). Virtually all of the liberated radioactivity was recovered in the neutral lipid fraction, mainly as diglyceride. These data show clearly that [2-^{3}H]acetate and [2-^{3}H]glycerol are specifically incorporated into the fatty acid and glycerol moieties, respectively, of the phospholipids, and that any loss of radioactivity from the phospholipid of cells preincubated with [2-^{3}H]glycerol is attributable to the turnover of *only* the glycerol moiety of the phospholipid.

To determine whether the stability of the phospholipid carbon was a peculiar feature of the acyl chains only, cells were incubated simultaneously with [*methyl*-^{14}C]thymidine and [2-^{3}H]glycerol, and then transferred to fresh unlabeled medium. The [2-^{3}H]glycerol content of cell phospholipid decreased more rapidly during the first 24 hr after transfer to unlabeled medium, than did the [^{14}C]DNA content (Figure 3A). In contrast to the studies on ^{3}H-labeled phospholipid acyl groups, however, the decrease in [2-^{3}H]glycerol phospholipid radioactivity over the remainder of the 8-day period continued at a rate in excess of that expected on the basis of dilution of label resulting from cell division alone

Table III: [2-^{3}H]Acetate and [2-^{3}H]Glycerol as Specific Precursors of the Acyl and Glycerol Moieties of L-Fibroblast Phospholipid.[a]

Source of Phospholipid Radioactivity	Treatment	Distribution of Radioactivity in Phospholipid Moieties after Treatment[b] (%)			
		Fraction of Phospholipid Hydrolyzed	Water Soluble	Fatty Acid and Fatty Acid Methyl Ester	Glycerol Containing Neutral Lipids[c]
[2-^{3}H]Acetate	Alkaline methanolysis	80	0.5	99.5	
[2-^{3}H]Glycerol	Alkaline methanolysis	99	99.0	1.0	
[2-^{3}H]Glycerol	Phospholipase C	80	1.7		97

[a] Radiolabeled lipid from 35×10^6 cells incubated for 48 hr with 50 μCi of either [2-^{3}H]acetate or [2-^{3}H]glycerol was subjected to alkaline methanolysis or treatment with phospholipase C. [b] Percent of total phospholipid radioactivity in lipid-soluble and water-soluble products was measured. For details of procedure, see Materials and Methods. [c] Nearly all of the radioactivity in the neutral lipids was associated with the diglycerides.

Table IV: Differences in Retention of Radiolabeled Acyl Groups and Glycerol in the Phospholipids of L-Cells.[a]

Days after Transfer to Unlabeled Medium	Source of Phospholipid Radioactivity			
	[2-^{3}H]Acetate[b]		[2-^{3}H]Glycerol[b]	
	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylethanolamine
0	50.3 ± 1.5	21.7 ± 2.4	60.8 ± 2.7	20.2 ± 1.5
1	48.3 ± 1.6	22.4 ± 3.7	50.4 ± 0.5	31.0 ± 0.6
2	45.7 ± 1.3	24.4 ± 3.5	41.6 ± 0.8	39.9 ± 1.5
4	44.9 ± 1.9	27.9 ± 2.6	32.8 ± 1.0	55.6 ± 0.8
6	42.3 ± 2.3	27.5 ± 2.3	28.8 ± 0.9	58.4 ± 1.5
8	44.4 ± 1.7	27.5 ± 3.8	26.3 ± 1.0	59.8 ± 0.4

[a] Cells incubated for 48 hr with 50 μCi of either [2-^{3}H]acetate or [2-^{3}H]glycerol were transferred to fresh unlabeled medium. At the indicated intervals radioactivity in the phosphatidylcholine and phosphatidylethanolamine fractions of the cells was determined. For details see Materials and Methods. [b] Results are given as the mean ±SD of four experiments.

(Figure 3A and Table I). It was subsequently found that the net rate of *loss* of radiolabeled glycerol from the phospholipid could be increased twofold if 0.1 m*M* glycerol (unlabeled) was included in the medium during the 8-day depletion period.

The rate of [2-^{3}H[glycerol depletion from the two principal phospholipid classes, phosphatidylcholine and phosphatidylethanolamine, was determined (Figure 3B). The content per 10^6 cells of [2-^{3}H]glycerol-labeled phosphatidylcholine and phosphatidylethanolamine decreased at a rate 1.37 and 1.12 as great, respectively, as that of the [^{14}C]DNA content per 10^6 cells. In the presence of 0.1 m*M* glycerol these rates increased to 1.44 and 1.24. As a consequence of the apparent differential lability of the acyl–glycerol bond, the ratio [2-^{3}H]glycerol-labeled phosphatidylcholine:[2-^{3}H]glycerol-labeled phosphatidylethanolamine decreased throughout the depletion period (Table IV). These data are in contrast to those obtained when the phospholipid acyl groups were radiolabeled during preincubation with [2-^{3}H]acetate, while there was a small decrease (6%) in the radiolabeled acyl group content of phosphatidylcholine and a corresponding increase in the phosphatidylethanolamine fraction during the first 2–4 days; no further changes in the distribution of radiolabel between the two classes were noted.

Retention of cellular phospholipid ^{3}H-acyl groups was not changed by including 0.1 m*M* sodium acetate or exogenous fatty acid in the medium during the depletion (Figure 4). The phospholipid ^{3}H-acyl group content of cells grown in a medium containing nontoxic levels (8.8 μM) of an equimolar mixture of oleic and linoleic acid decreased at a rate paralleling that for the depletion of [^{14}C]DNA (Table I). Gas chromatographic analysis of the phospholipid from those cells showed that there was enough of the exogenous fatty acid present to cause a tenfold increase in linoleic acid content (Table V). Lipid soluble phosphorus determinations (Rouser et al., 1970) showed that the total cellular phospholipid content per cell was unchanged by the presence of the exogenous lipids. The virtual absence of polyunsaturated fatty acids in the zero-time phospholipid is consistent with other data (Geyer et al., 1962) which show that the L-fibroblast lipid contains these fatty acids only when they are supplied in the media. That very significant quantities of exogenous fatty acids entered the cells was indicated by the presence of numerous cytoplasmic lipid droplets revealed by phase contrast microscopy. In earlier studies (Schneeberger et al., 1971) the composition of these droplets was shown to be triglyceride composed primarily of the exogenously supplied fatty acids. It was further shown that during their subsequent metabolism these triglycerides gave rise in turn to free fatty acids. These results clearly demonstrate that in spite of prolonged exposure to elevated intracellular fatty acid concentrations, the complete retention of phospholipid acyl groups was unaffected.

Discussion

Phospholipids are important components of all cellular membranes. Recent data have shown that altering the fatty acid composition of the cellular phospholipids may lead to alterations in the fluidity of the plasma membrane (Linden et al., 1973; Rittenhouse et al., 1974), and that these changes may in turn alter the structural (Pisetsky and Terry, 1972) or biochemical (Rittenhouse et al., 1974; Ferguson et al., 1975) properties of the membrane. Evidence for the turnover of different portions of the phospholipid molecule at different rates has been reviewed (Hill and Lands, 1970). Of particular interest are data which demonstrate the turnover of phospholipid acyl groups in such diverse systems as liver (Holub et al., 1971; Pisetsky and Terry, 1972), erythrocytes (Shohet et al., 1968), lymphocytes (Resch et al., 1972, Ferber and Resch, 1973), and lung (Tierney et al., 1967). Limited data are available concerning the relative retention or loss of various portions of the phospholipid molecule in cells cultured in vitro (Geyer,

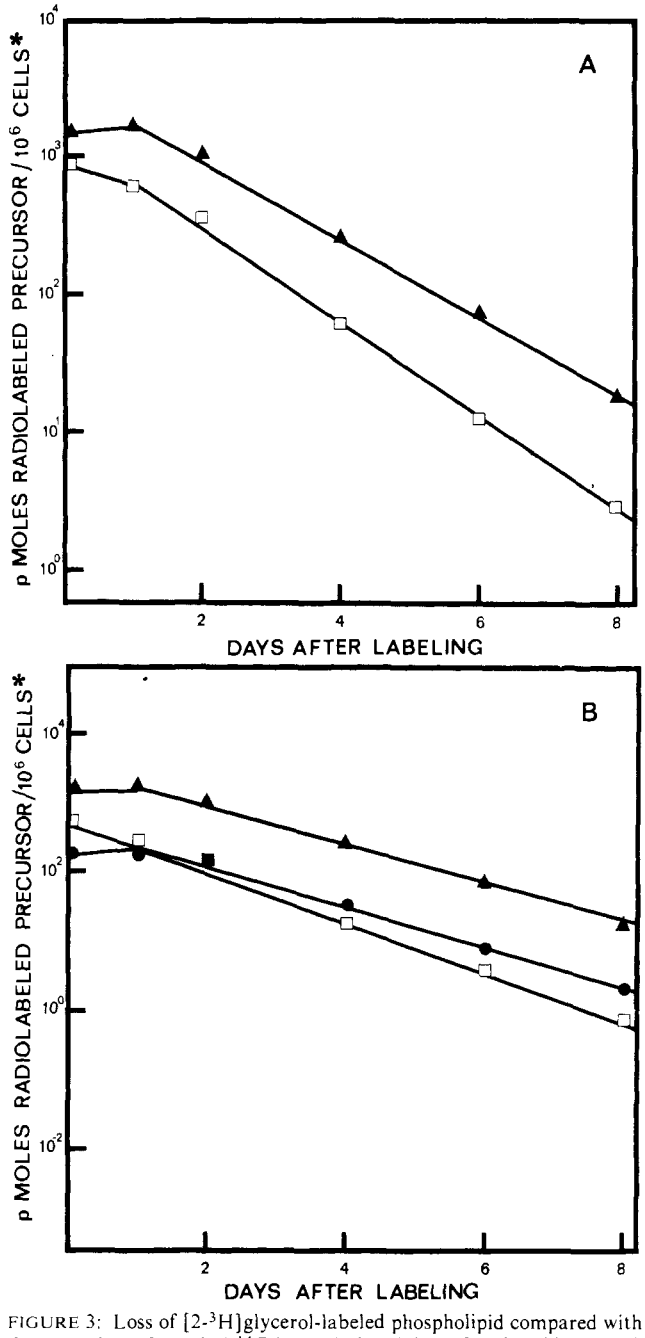

FIGURE 3: Loss of [2-^{3}H]glycerol-labeled phospholipid compared with the retention of *methyl*-^{14}C-labeled thymidine of L-fibroblasts. (A) Cells incubated with a mixture of 5 μCi of [*methyl*-^{14}C]thymidine and 50 μCi of [2-^{3}H]glycerol for 48 hr were transferred to nonradioactive medium. At the times shown the [^{14}C]DNA and total ^{3}H-labeled phospholipid content per 10^6 cells were determined. All points are the means of data from three experiments. Statistical evaluation of the slopes of the regression lines calculated using data from days 1 to 8 are given in Table I. (▲) [*methyl*-^{14}C]Thymidine-labeled DNA; (□) total [2-^{3}H]glycerol-labeled phospholipid. (B) Same as in (A) except that the phospholipid was fractionated by thin-layer chromatography and the radioactivity of phosphatidylcholine and phosphatidylethanolamine was measured. All points are the means obtained from three experiments. (▲) [*methyl*-^{14}C]Thymidine-labeled DNA; (●) [2-^{3}H]glycerol-labeled phosphatidylethanolamine; (□) [2-^{3}H]glycerol-labeled phosphatidylcholine. For graphical presentation the picomoles of [2-^{3}H]glycerol-labeled phospholipid/10^6 cells was multiplied by 10.

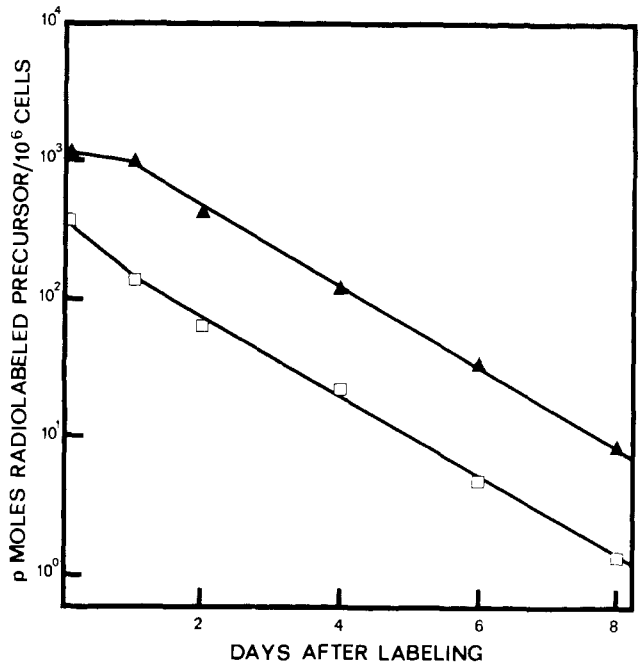

FIGURE 4: Availability of exogenous fatty acid and retention of radiolabeled phospholipid acyl groups by L-fibroblasts. Cells were incubated with a mixture of 50 μCi of [2-^{3}H]acetate and 5 μCi of [*methyl*-^{14}C]thymidine, and then transferred to a nonradioactive medium containing an equimolar mixture of oleic and linoleic acids (8.8 μm). This mixture was added to the cultures daily. Aliquots of the cells were analyzed, at the times shown, for their content of radiolabeled phospholipid and DNA. Each point is the mean of three determinations. (▲) [*methyl*-^{14}C]thymidine-labeled DNA; (□) [2-^{3}H]acetate-labeled phospholipid. Statistical evaluation of the slopes from day 1 through 8 of the depletion is given in Table I.

1967; Warren and Glick, 1968; Peterson and Rubin, 1969; Pasternak and Bergeron, 1970). Results from experiments with strain L-fibroblasts, however, show that phospholipid *acyl group turnover is not a general phenomenon* found in all mammalian cells. After a small loss (10%) occurring within 24–36 hr after transfer to unlabeled medium, phospholipid ^{3}H-labeled fatty acids are retained to the same extent as [*methyl*-^{14}C]thymidine-labeled DNA. Preliminary experiments with HeLa cells and human conjunctival cells (R. P. Geyer, unpublished experiments) suggest that the "irreversible" incorporation of fatty acid into cellular phospholipid is not confined to fibroblasts.

The lability of the acyl-glycerol bond as estimated by the turnover rate of phospholipid glycerol shows that retention of phospholipid acyl groups occurs in spite of deacylation-reacylation reactions. Although the factors which are, by their presence or absence, responsible for the complete retention of phospholipid fatty acid remain to be defined, there is evidence which suggests that phospholipid fatty acids are metabolically distinct from and do not equilibrate with other extra- or intracellular sources of acyl groups. This evidence is summarized as follows. (1) The ratio of ^{3}H-acyl group radioactivity in the phosphatidylcholine fraction:^{3}H-acyl group radioactivity in the phosphatidylethanolamine fraction is unchanged from the first through eighth day of the isotope depletion period (Table III). From estimates of glycerol turnover rate the phosphatidylcholine acyl-glycerol bond lability is three times that of phosphatidylethanolamine (Figure 3B). Under these conditions, if unesterified fatty acid produced by cleavage of the acyl-glycerol bond had equilibrated with a fatty acid pool used for de novo phospholipid biosynthesis, a shift in the content of ^{3}H-acyl groups from the phosphatidylcholine to phosphatidylethanolamine would have been expected. (2) In lipid-free medium strain L-fibroblast lipids are devoid of polyunsaturated fatty acids (Geyer et al., 1962). During preincubation with [2-^{3}H]acetate, therefore, only ^{3}H-la-

Table V: Effects of Exogenous Oleic and Linoleic Acid on the Phospholipid Fatty Acid Composition of Strain L-Fibroblasts.[a]

Days after Transfer	Composition of Phosphoglyceride Fatty Acids (% by Weight)						
	14:0[b]	16:0	16:1	18:0	18:1	18:2	18:3
0	0.8 ± 0.6	16.1 ± 0.9	7.4 ± 2.2	9.0 ± 0.5	61.6 ± 3.2	1.9 ± 0.6	2.4 ± 1.4
1	0.4 ± 0.2	13.6 ± 0.5	2.7 ± 0.2	9.4 ± 0.4	55.4 ± 0.9	16.4 ± 0.6	2.1 ± 0.1
4	0.7 ± 0.2	13.4 ± 1.1	2.9 ± 0.4	9.3 ± 0.4	45.9 ± 1.2	26.5 ± 0.3	1.4 ± 0.1
6	0.5 ± 0.2	12.1 ± 2.7	1.8 ± 0.6	11.6 ± 0.8	45.2 ± 1.7	26.7 ± 0.4	2.0 ± 0.8
8	N.D.[c]	12.0 ± 5.3	2.9 ± 1.8	12.9 ± 0.4	48.6 ± 5.9	21.1 ± 0.8	2.7 ± 0.6

[a] Radiolabeled phospholipid from cells incubated with [2-^{3}H]acetate and transferred to unlabeled medium, as described in Figure 4 was methylated, and the fatty acid composition determined by gas chromatography as described under Materials and Methods. All values are the mean ±SD of four separate experiments. [b] Fatty acid nomenclature: number of carbons: number of double bonds. [c] Not determined.

beled saturated and monoenoic acids are available for biosynthesis of phosphoglycerides. In deacylation–reacylation reactions, however, polyenoic acids are preferentially incorporated, at least into the 2 position of phosphoglycerides (Lands and Merkel, 1963). If exogenously added polyenoic fatty acids equilibrate with a pool of saturated or monoenoic ^{3}H-fatty acid produced during hydrolysis of the acylglycerol bond, their presence during the 8-day isotope depletion period should cause a decrease in the reutilization of the isotopically labeled fatty acid. As shown in Figure 4, no such change in retention of the radiolabel was observed when cells preincubated with [2-^{3}H]acetate were subsequently maintained for 8 days in medium supplemented with a mixture of oleic and linoleic acids. It is important to note that the intracellular availability of exogenous acid during this time was sufficient to cause a tenfold increase, presumably by de novo synthesis, of the linoleic acid content of the phospholipid (Table V) and to stimulate the formation of cytoplasmic triglyceride droplets.

It should be emphasized that the retention of acyl groups is not an attribute of all cellular lipids. In fact, earlier work showed that the acyl groups incorporated into L-fibroblast triglycerides interchange rapidly within the cell, and are lost from the cell at a rate which is too rapid to be accounted for by cell division alone (Schneeberger et al., 1971). Preliminary data show that these acyl groups may be utilized for phospholipid biosynthesis, or that they may be lost into the medium. Losses of acyl groups from the triglyceride fraction into the medium are dependent on there being a suitable acyl group acceptor, e.g., serum protein present in the medium. The protein content of the medium has no effect, however, on the retention of phospholipid acyl groups (Geyer, 1967).

Warren and Glick (1968) showed that during the log phase of growth, the rate of loss of total lipid soluble radioactivity from the surface membrane of strain L-fibroblasts slightly exceeded the rate of cell multiplication, and that culturing these cells at high population densities accelerated this loss. Since the precursor used was [U-^{14}C]glucose, the loss of lipid-soluble radioactivity occurring during the log phase of growth probably resulted from the incorporation of glucose carbon into both the acyl group *and* the *glycerol* of cell lipids. The discrepancy between these data and those of Geyer (1967) is explained by the differential turnover of glycerol relative to the acyl groups, as demonstrated in the present study. It was suggested (Warren and Glick, 1968) that accelerated rates of loss observed when cells were maintained at high population densities demonstrated a relationship between the rate of multiplication and retention of phospholipid acyl groups. To what extent problems associated with the maintenance of viable cells at high densities (Eidam and Merchant, 1965a,b) might have contributed to these results is uncertain. Geyer (1967), however, observed no difference between the rate of phospholipid acyl group turnover in cells grown in serum-supplemented vs. serum-free medium, despite the fact that the growth rate in the latter is one-half to one-third that in the former.

The small loss of radioactive acyl groups which occurs during the first 24 hr of the depletion period may result from there being two pools of phospholipid. Immediately after de novo synthesis a given phospholipid molecule as part of a labile pool may be subject to degradation, its acyl group being either oxidized, incorporated into triglyceride, or lost to the culture medium. Alternatively the phospholipid can be used in the biosynthesis of cellular membranes. In the latter instance the molecule has entered a pool in which, despite deacylation–reacylation reactions, the group cannot be transferred back to the cytoplasm or into the medium. Additional experiments are in progress to determine factors which mediate the exchange of phospholipid acyl groups between these pools.

Whatever the mechanism underlying this phenomenon, it offers a rationale for another experimental approach to the modification of the membrane acyl groups in mammalian cells (Linden et al., 1973; Rittenhouse et al., 1974). Since once incorporated into the membrane a fatty acid is completely retained, it should be possible, by supplying specific fatty acids in the medium, to cause enrichment of that fatty acid within the phospholipids without using inhibitors of fatty acid biosynthesis (Williams et al., 1974). Preliminary experiments in this laboratory have shown that with the careful addition of small amounts of polyunsaturated fatty acids to the culture medium, as much as 50–60% of the cellular phospholipid fatty acid can be derived from the exogenous fatty acid. Such cells can be maintained in culture for at least 2 weeks, with only a slight decrease in their rate of multiplication.

Acknowledgments

The authors express their appreciation to Ms. Deborah Weil and Mrs. Joanne McCormack for their excellent technical assistance.

References

Albutt, E. C. (1966), *J. Med. Lab. Technol. 23,* 61.

Allman, D. W., Hubbard, D. D., and Gibson, D. M. (1965), *J. Lipid Res. 6,* 63.

Dhopeshwarkar, G. A., Subramanian, C., and Mead, J. F. (1971), *Biochim. Biophys. Acta 239,* 162.

Eidam, C. R., and Merchant, D. J. (1965a), *Exp. Cell Res. 37,* 132.
Eidam, C. R., and Merchant, D. J. (1965b), *Exp. Cell Res. 37,* 147.
Ferber, E., and Resch, K. (1973), *Biochim. Biophys. Acta 296,* 335.
Ferguson, K. A., Glaser, M., Bayer, W. H., and Vagelos, P. R. (1975), *Biochemistry 14,* 146.
Folch, J., Lees, M., and Stanley, G. H. S. (1957), *J. Biol. Chem. 226,* 497.
Geyer, R. P. (1967), in Lipid Metabolism in Tissue Culture Cells, Rothblatt, G. H., and Kritchevsky, D., Ed., Philadelphia, Pa., The Wistar Institute Press, p 33.
Geyer, R. P., Bennett, A., and Rohr, A. (1962), *J. Lipid Res. 3,* 80.
Glass, R. L. (1971), *Lipids 6,* 919.
Hajra, A. K., Sequin, E. B., and Agranoff, B. W. (1968), *J. Biol. Chem. 243,* 1609.
Hill, E. E., and Lands, E. M. (1970), in Lipid Metabolism, Wakil, S. J., Ed., New York, N.Y., Academic Press, p 185.
Holub, B. J., Breckenridge, W. C., and Kuksis, A. (1971), *Lipids 6,* 307.
Kaulen, H. D. (1972), *Anal. Biochem. 45,* 664.
Kennedy, E. P., and Weiss, S. B. (1956), *J. Biol. Chem. 222,* 193.
Krause, R. F., and Beamer, K. C. (1972), *J. Nutr. 102,* 1465.
Lands, W. E. M. (1960), *J. Biol. Chem. 235,* 2233.
Lands, W. E. M., and Merkel, J. (1963), *J. Biol. Chem. 238,* 898.
Lee, T. C., Stephens, N., Moehl, A., and Snyder, F. (1973), *Biochim. Biophys. Acta 291,* 86.
Linden, C. D., Wright, K., McConnell, H. M., and Fox, C. F. (1973), *Proc. Natl. Acad. Sci., U.S.A. 70,* 2271.
Marinetti, H. V., Erbland, J., and Stotz, E. (1958), *J. Biol. Chem. 233,* 562.
Mavis, R. D., Bell, R. M., and Vagelos, P. R. (1972), *J. Biol. Chem. 247,* 2835.
Pasternak, C. A., and Bergeron, J. J. M. (1970), *Biochem. J. 119,* 473.
Peterson, J. A., and Rubin, H. (1969), *Exp. Cell Res. 58,* 365.
Pisetsky, D., and Terry, T. M. (1972), *Biochim. Biophys. Acta 274,* 95.
Reed, C. F. (1968), *J. Clin. Invest. 47,* 749.
Resch, K., Gelfund, E. W., Hansen, Käthe, and Ferber, E. (1972), *Eur. J. Immunol. 2,* 598.
Rittenhouse, H. G., Williams, R. E., Wisnieski, B., and Fox, C. F. (1974), *Biochem. Biophys. Res. Commun. 58,* 222.
Rouser, G., Fleischer, S., and Yamamoto, A. (1970), *Lipids 5,* 494.
Schneeberger, E. E., Lynch, R. D., and Geyer, R. P. (1971), *Exp. Cell Res. 69,* 193.
Shohet, S. B., Nathan, D. G., and Karnovsky, M. L. (1968), *J. Clin. Invest, 47,* 1096.
Skipski, V. P., Peterson, R. F., Sanders, J., and Barclay, M. (1963), *J. Lipid Res. 4,* 227.
Tierney, D. F., Clements, J. A., and Trahan, H. J. (1967), *Am. J. Physiol. 213,* 671.
Warren, L., and Glick, M. C. (1968), *J. Cell. Biol. 37,* 729.
Webb, R. A., and Mettrick, D. F. (1972), *J. Chromatogr. 67,* 75.
Williams, R. E., Wisnieski, B. J., Rittenhouse, H. G., and Fox, C. F. (1974), *Biochemistry 13,* 1969.