Intracellular Phospholipid Movement and the Role of Phospholipid Transfer Proteins in Animal Cells†

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ABSTRACT: The mechanism of the intracellular movement of phospholipids from their site of synthesis in the endoplasmic reticulum to mitochondria and other cell membranes is a major unsolved problem of cell biology. Phospholipid transfer proteins of varying specificity found in the soluble supernatant fractions of many tissues catalyze the transfer of phospholipids from microsomes to mitochondria in vitro. They are postulated to play a similar role in vivo, but evidence for their function in living cells is lacking. We have now used an analogue of choline, *N*-propyl-*N*,*N*-dimethylethanolamine [PDME, (2-hydroxyethyl)dimethylpropylammonium hydroxide], to devise a test for the function of the transfer proteins in living cells. The rates of translocation of newly synthesized phosphatidylcholine and the analogue phosphatidyl-PDME in living cells were compared with the rates of transfer in vitro catalyzed by soluble transfer proteins extracted from the same cells. Labeled PDME, choline, and ethanolamine were found to be rapidly incorporated into the lipids of isolated rat hepatocytes and of baby hamster kidney (BHK-21) cells in culture. The translocation of newly synthesized phosphatidylcholine and phosphatidyl-PDME was very rapid in both types of cells with a half-time for equilibration of a few minutes, while the translocation of phosphatidylethanolamine was much slower, with a half-time 20–80 fold longer than those of the other two phospholipids. We then compared these relative rates of movement with the activities of the phospholipid transfer proteins of the respective cells. Partially purified phosphatidylcholine transfer protein from rat liver transfers phosphatidylcholine and phosphatidyl-PDME at identical rates but transfers phosphatidylethanolamine at a rate too low to be detected. This result is consistent with an essential function of this transfer protein in vivo. In contrast, partially purified phosphatidylcholine phospholipid transfer protein from BHK cells transfers phosphatidylcholine rapidly, while no transfer of phosphatidyl-PDME and phosphatidylethanolamine was detected. We further found that the specific phosphatidylcholine transfer protein of BHK cells accounts for nearly all of the transfer activity detected in the crude soluble fraction. The rapid translocation of phosphatidyl-PDME in vivo in BHK cells is therefore inconsistent with the postulate that soluble phospholipid transfer proteins are responsible for the rapid movement of phospholipids from microsomes to mitochondria in living cells.

The final steps in the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine, the major phospholipids in mammalian cells, occur in the endoplasmic reticulum (Wilgram & Kennedy, 1963; Dawson, 1973). Membrane biogenesis and lipid turnover in other organelles require the transfer of phospholipids from the endoplasmic reticulum to these other subcellular membranes. The mechanism and control of this transfer are as yet unknown.

The movement of phospholipids between microsomes and mitochondria has been demonstrated in cell-free systems (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969; Akiyama & Sakagami, 1969). This transfer is greatly accelerated by phospholipid transfer proteins of varying specificity found in the soluble cytosol fraction of many tissues (Wirtz, 1974; Wirtz & Van Deenen, 1977). Such proteins have been postulated (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969) to function in vivo to facilitate the movement of phospholipids between subcellular membranes in the cell, but direct evidence for such a function has been lacking.

In the present study we have devised a test for the possible functioning of phospholipid transfer proteins in isolated rat hepatocytes and in baby hamster kidney (BHK-21) cells in culture, by use of an analogue of choline, *N*-propyl-*N*,*N*-dimethylethanolamine [PDME,[1] (2-hydroxyethyl)dimethylpropylammonium hydroxide]. We have found that the labeled bases choline, PDME, and ethanolamine are rapidly incorporated into microsomal phospholipids in both cell types. We have characterized the intracellular transfer of these newly made phospholipids from endoplasmic reticulum to mitochondria by examining the labeling of lipids in subcellular fractions isolated after incubation of the cells with radiolabeled precursors. We have further compared the intracellular pattern of phospholipid movement with the transfer of these same phospholipids by phospholipid transfer proteins in a cell free system.

Experimental Procedures

Hepatocyte Preparation. Male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 150–300 g were fed ad libitum. Hepatocytes were isolated and parenchymal cells purified by the method of Seglen (1973) with the following modifications. Perfusion was performed in situ at rates of 35–40 mL/min, the collagenase perfusion was performed for 20–30 min, and the final Hepes–Tes–Tricine suspension buffer was supplemented with 2% (w/v) bovine serum albumin containing 1 mM oleic acid by the technique of Spector & Hoak (1963). Collagenase type IV, obtained from Sigma, was used for all cell preparations. Cell viability

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[1] Abbreviations: PDME, *N*-propyl-*N*,*N*-dimethylethanolamine, (2-hydroxyethyl)dimethylpropylammonium hydroxide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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following isolation was 80–90% as measured by trypan blue exclusion. Cells were held at 0–4 °C until initiation of incorporation studies.

Tissue Culture. Baby hamster kidney (BHK-21) cells (ATCC CCL10) were grown in Delbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), 5% fetal bovine serum (MA Bioproducts), and 5% calf serum (MA Bioproducts). The same media free of choline were made in the laboratory from amino acids, vitamins, and salts.

Synthesis of Propyldimethyl[^{3}H]ethanolamine. Propyldimethyl[^{3}H]ethanolamine was synthesized by reductive methylation of ethanolamine with formaldehyde and sodium cyanoborohydride to dimethylethanolamine, followed by propylation of the dimethylethanolamine with propyl iodide.

The reductive methylation, based on the general procedure of Borch et al. (1971), was performed by treating [1-^{3}H]-ethanolamine hydrochloride (10–23 Ci/mmol, Amersham) with 0.1 M formaldehyde and 0.04 M $NaCNBH_4$ in a total volume of 1.0 mL of 0.2 M sodium borate buffer, pH 9.0. The mixture was vigorously shaken at 30 °C for 2.5 h, after which 4 drops of 2 M HCl was added, and the mixture was heated in a boiling water bath for 5 min. The mixture was cooled to room temperature and applied to a column (58 × 1.1 cm) of AG-50 resin, H^+ form, 100–200 mesh (Bio-Rad). The column was washed with 165 mL of H_2O. Dimethylethanolamine was eluted with 1.5 M HCl and emerged from the column after approximately 220 mL of wash. The pooled fractions containing the product were taken to dryness in a rotary evaporator. The residue was taken up in H_2O and dried again. The product was then dissolved in a minimal volume of methyl ethyl ketone and neutralized with a small volume of NaOH.

Propylation of dimethylethanolamine was carried out by treating the dimethyl[^{3}H]ethanolamine with 0.5 M propyl iodide in a total of 0.2 mL of methyl ethyl ketone in a sealed MiniAktor tube (Applied Sciences) at 70 °C for 16 h. The reaction mixture was then mixed with 2.0 mL of H_2O and applied to a column (55 × 1.1 cm) of Amberlite CG-50, 100–200 mesh, ammonium form (Mallinckrodt). The column was washed with 260 mL of H_2O, and the product eluted with an 800-mL linear gradient of 0–0.2 M ammonium acetate, pH 9.5. The product was eluted at approximately 64 mM ammonium acetate. The pooled fractions were taken to near dryness in a rotary evaporator at 50 °C. The residue was further dried in a vacuum over H_2SO_4 and solid KOH for 1 week. The product was taken up in H_2O, dried again in a rotary evaporator, and finally dissolved in a desired volume of H_2O.

The propyldimethyl[^{3}H]ethanolamine was identified by thin-layer chromatography on silica gel G plates (Supelco) activated at 110 °C for 1 h and run in a system composed of 50 g of phenol, 50 mL of 1-butanol, 3 mL of 80% formic acid, and 10 mL of H_2O, with the total mixture saturated with solid KCl. The product chromatographed with an R_f of 0.33. The identity of the product was further confirmed by field desorption mass spectrometry which showed a molecular ion at m/z 132 and indicated the presence of less than 1% of any contaminating, quaternized products.

Preparation of Radiolabeled Phospholipids and Liposomes. Phosphatidyl[^{14}C]choline, phosphatidyl[^{3}H]ethanolamine, and phosphatidylpropyldimethyl[^{3}H]ethanolamine were prepared biosynthetically by growing BHK-21 cells in the presence of the corresponding radiolabeled phospholipid base. Cells were plated at 2 × 10^6/60-mm dish 48 h prior to the addition of the radiolabeled compound. [^{14}C]Choline (New England Nuclear, 46 μM, 54 mCi/mmol) and propyldimethyl[^{3}H]-ethanolamine (2 μM, 23 Ci/mmol) were added to the cells in choline-free media, and cells were grown for an additional 16 h. [1-^{3}H]Ethanolamine (Amersham, 5 μM, 23 Ci/mmol) was added to the cells in complete medium, and cells were subsequently grown for 6 h. Following incubations, the cells were harvested in phosphate-buffered saline with a rubber policeman. Phospholipids were extracted from the cells by the method of Bligh & Dyer (1959). The phospholipid species were separated by thin-layer chromatography on silica gel G plates (Supelco) activated at 110 °C for 30 min and run in a system of chloroform–methanol–acetic acid–water (50:25:8:2.5). Phospholipids were detected with iodine vapor. Areas corresponding to the labeled species were scraped from the plates, and the lipids were eluted with chloroform–methanol–ammonium hydroxide (20:10:1). Phospholipids were stored in chloroform–methanol (9:1) at −20 °C.

Phosphatidylcholine liposomes containing phosphatidyl-[^{14}C]choline and phosphatidyl[^{3}H]ethanolamine, phosphatidyl[^{14}C]choline and phosphatidylpropyldimethyl[^{3}H]-ethanolamine, or phosphatidyl[^{3}H]choline and [^{14}C]triolein (New England Nuclear) were prepared by a procedure modified from that described by Bloj & Zilversmit (1977). Gg phosphatidylcholine (94 mg) in chloroform was mixed with less than 1 mg of radiolabeled phospholipid and 48 μg of butylated hydroxytoluene. The mixture was dried in a stream of N_2, and the residue was dissolved in 5 mL of diethyl ether. The mixture was again dried, and the lipids were suspended in 2 mL of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, and 0.5% NaN_3, pH 7.4, by gentle agitation under N_2. The suspension was held at room temperature under N_2 for 1 h. The lipids were sonicated under N_2 in a bath sonicator (Branson) at a temperature under 15 °C until a clear solution was achieved (20–40 min). The liposomes were then twice frozen in a dry ice–propanol freezing bath and thawed. This treatment was found to yield liposomal preparations which gave lower backgrounds by reducing fusion or adsorption of liposomes with acceptor mitochondria in phospholipid transfer assays. The patterns of specificity of phospholipid transfer from liposomes prepared with or without this treatment were identical. Liposomes were finally centrifuged at 12000g for 15 min at 0–4 °C. The pellet was discarded, and the liposomes in the supernatant were stored under N_2 at 0 °C.

Incorporation of Labeled Lipid Precursors. Freshly isolated hepatocytes were first incubated in Hepes–Tes–Tricine suspension buffer (Seglen, 1973) with 10% albumin–oleate and glucose (2 g/L) in Erlenmeyer flasks for 15 min at 37 °C in a rotary water bath (New Brunswick Scientific) following a 10-cm circular path and operating at 90 rpm. Radioactive precursors [[*methyl*-^{3}H]choline chloride (1.3 mCi/mmol), [*methyl*-^{14}C]choline chloride (54 mCi/mmol), [*methyl*-^{14}C]methionine (6.3 mCi/mmol), propyldimethyl[1-^{3}H]-ethanolamine (23 Ci/mmol), or [1-^{3}H]ethanolamine (2.6 mCi/mmol or 23 Ci/mmol)] were next added in suspension buffer, and incubations were continued at 37 °C. All radiolabeled compounds were purchased from New England Nuclear except for [1-^{3}H]ethanolamine which was obtained from Amersham and propyldimethyl[1-^{3}H]ethanolamine which was prepared as described above. The concentrations of precursors in the incubation mixtures were 1.0 mM or 3–6 μM choline chloride, 0.25 mM methionine, 0.25 mM ethanolamine, and 1.0 mM or 0.15 μM PDME. Periodically, samples of incubation mixture were removed for cell fractionation or lipid extraction.

For chase experiments, incubation mixtures were diluted 4-fold with Hepes–Tes–Tricine suspension buffer containing albumin–oleate, glucose (2 g/L), and unlabeled lipid precursor at concentrations 5-fold greater than the originally present in the pulse-labeling incubation mixture. The diluted mixture was further incubated at various temperatures. Portions were periodically removed for cell fractionation or lipid extraction.

Cell viability during incubations was assessed by the ability of the cells to exclude trypan blue. No incubation resulted in a decrease in viable cell number of more than 1%.

As a further test of their metabolic integrity, the ability of cells to initiate new phospholipid synthesis during prolonged chase experiments was assessed by measuring initial rates of incorporation of [*methyl*-^{14}C]choline into cell phospholipid. Small samples of cells were removed at various times and treated with [*methyl*-^{14}C]choline for short intervals. The cellular lipids were then extracted, and the [*methyl*-^{14}C]choline incorporation was examined.

Incorporation studies with BHK-21 cells were performed on cells plated at 2×10^6/60-mm dish 48 h prior to addition of radiolabeled compounds. Cells were washed with phosphate-buffered saline (37 °C), and incorporation was initiated by addition of 2 mL of medium containing radiolabeled lipid precursors [[^{14}C]choline (54 mCi/mmol), [^{3}H]PDME (23 Ci/mmol), or [^{3}H]ethanolamine (23 Ci/mmol)]. The concentrations of propyldimethyl[^{3}H]ethanolamine and [^{3}H]-ethanolamine in the incubation mixtures were 0.5–1.0 μM, while [^{14}C]choline was added to a concentration of 2–6 μM. Separate, identical dishes of cells were used for each time point. All incorporations were performed on duplicate sets of dishes.

Cell Fractionations. Portions of hepatocyte incubation mixtures were diluted 6-fold with ice-cold Hepes wash buffer (Seglen, 1973). This manipulation halted all precursor incorporation within 30 s. All subsequent steps were carried out at 0–4 °C. Cells were pelleted by low speed centrifugation, resuspended in ice-cold STE buffer (250 mM sucrose, 10 mM Tris-HCl, and 1.0 mM EDTA, pH 7.4) and homogenized for 90 s with a Potter-Elvehjem homogenizer in an ice–water jacket.

The crude homogenate was centrifuged at 2250 rpm (60*g*) for 10 min in an SS-34 rotor in a Sorvall RC2B centrifuge. Precipitate I, containing nuclei and debris, was discarded. Supernatant I was centrifuged at 8500 rpm (8700*g*) for 15 min yielding supernatant II, and precipitate II, the mitochondrial fraction. The mitochondrial pellet was washed 3 times by suspension in STE buffer with a Potter-Elvehjem homogenizer, followed by centrifugation at 8500 rpm for 15 min to yield the final washed mitochondrial fraction. Supernatant II was centrifuged at 10 000 rpm (12000*g*) for 10 min. The pellet containing a mixture of mitochondria and microsomes was discarded. Supernatant III was centrifuged at 40 000 rpm (105000*g*) for 60 min in a Beckman type 40 rotor in a Beckman ultracentrifuge. The resuspended pellet from this centrifugation constituted the microsomal fraction.

The possible transfer of phospholipid from one subcellular fraction to the other during cell fractionation was tested by mixing previously labeled isolated microsomes or mitochondria with unlabeled hepatocyte homogenate followed by reisolation of subcellular fractions. Less than 2% of the radioactivity added in microsomes was transferred to the mitochondrial fraction. When labeled mitochondria were added to the homogenate, less than 3% of the radioactivity was recovered in the microsomal fraction.

In studies where mitochondria were subfractionated, homogenates from portions of cell incubation mixtures were mixed with unlabeled rat liver homogenate (Wilgram & Kennedy, 1963) to provide enough material for subsequent fractionation. Mitochondria were then purified as described above. Outer membrane–intermembrane (OM) and inner membrane–matrix (IM) fractions were prepared by the digitonin-fractionation method of Greenawalt (1974).

The incorporation of lipid precursors into BHK-21 cells was stopped by removing the media by aspiration and washing the cells twice with ice-cold 0.15 M NaCl containing 2 mM EGTA, pH 7.4. Cells were then washed twice with ice-cold 0.25 M sucrose containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.4 (STE buffer), and harvested in the same buffer with a rubber policeman. The cells were pelleted by centrifugation at 4000 rpm for 1 min, 0–4 °C. They were then suspended in 10 mL of STE buffer containing unlabeled BHK-21 cells (2 g wet weight; ca. 6×10^8 cells) grown in roller bottles by the Massachusetts Institute of Technology Cell Culture Center. These unlabeled, carrier cells were harvested as described above for the labeled cells but were then resuspended in STE buffer and held on ice for 24 h prior to use. The mixed cells were homogenized for 15 s at 4 °C by using a Willems Polytron apparatus (Brinkmann) at a setting of 5.

Microsomes and mitochondria were isolated from the BHK cell homogenate as described above for hepatocytes with the following modifications. Pellet II (from the first 8500 rpm centrifugation) was composed of a loose portion and a tightly packed portion. The loose portion was removed with a Pasteur pipet, resuspended in ice-cold STE buffer, and centrifuged again at 8500 rpm for 15 min. The loose portion of the pellet from this centrifugation was discarded, while the tightly packed portion was resuspended and combined with the first tightly packed pellet. These combined mitochondrial pellets were then washed 4 times as described above. The pellet from the 40.000 rpm centrifugation was washed once by resuspension in STE buffer and centrifuged again at 40 000 rpm for 60 min. The resuspended pellet was then centrifuged at 10 000 rpm for 10 min. The supernatant from this centrifugation constituted the microsomal fraction.

Analysis of Phospholipids. Lipids were extracted by the procedure of Bligh & Dyer (1959).

Phospholipids in the chloroform extracts were separated by thin-layer chromatography on silica gel G (Supelco) or silica gel H (Applied Sciences) plates which had been activated by heating at 110 °C for 30 min. Plates used for the quantitative analysis of phospholipid species were first run in acetone to remove phosphorus-containing contaminants. Separation of phospholipids was performed in chloroform–methanol–acetic acid–water (50:25:8:2). Phosphatidylethanolamine from *Escherichia coli* and egg phosphatidylcholine were standards.

Phospholipid spots were detected by exposing the plates to iodine vapor (for later measurement of radioactivity) or by spraying plates with H_2SO_4 and heating in a vacuum oven at 160 °C (for later phosphate analysis).

Radioactivity in the chloroform extracts or thin-layer spots was determined by liquid scintillation counting. Chloroform from a portion of extract was mixed with 0.01 mL of 10% Triton X-100 in a scintillation vial, and the solvent was evaporated in a boiling water bath. Radioactivity was measured after addition of 0.5 mL of water and 5.0 mL of Liquiscint (National Diagnostics). Phospholipid spots from plates were scraped directly into scintillation vials and counted in 0.5 mL of water and 5.0 mL of Liquiscint.

Total phosphorus was measured in both chloroform extracts (after evaporation of solvent) and scraped spots by ashing samples with $Mg(NO_3)_2$ (Ames & Dubin, 1960) followed by

microdetermination of phosphate by the method of Chen et al. (1969).

For further identification, phospholipids were treated with phospholipase D (Sigma) as described by Kates & Sastry (1969) and the radioactive bases separated by thin-layer chromatography on silica gel G as described above for propyldimethylethanolamine.

Assay of Marker Enzymes. The purity of subcellular fractions was assessed by specific enzyme markers: NADPH–cytochrome *c* reductase for microsomes, succinate–cytochrome *c* reductase for mitochondria, monoamine oxidase for mitochondrial outer membrane, and cytochrome *c* oxidase for mitochondrial inner membrane. NADPH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were assayed as described by Sottocasa et al. (1967) except that the NADPH-dependent reaction was run in the presence of 0.1% deoxycholate, the succinate-dependent reaction was performed with 0.03% deoxycholate, and both reactions were performed in a total volume of 1.0 mL. Monoamine oxidase was measured by the method of Wurtman & Axelrod (1963). Cytochrome oxidase was assayed by the procedure of Wojtczak et al. (1972) except that the reaction was carried out in 125 mM potassium phosphate buffer, pH 7.4, and 1% Triton X-100. Protein was determined by the method of Lowry et al. (1951) except for measurements taken during subfractionation of mitochondria which were made with the biuret reagent described by Gornall et al. (1949).

Contamination of one subcellular fraction by another was calculated from the specific activities of a given contaminating marker enzyme. The specific activities of phospholipids in mitochondrial fractions were corrected for contamination by microsomes. The correction was based on the measured contamination derived from measurement of marker enzymes, as described above, the amount and radioactivity of the lipids in each purified fraction, and the phospholipid to protein ratio of the subcellular fractions. For example, if the specific activity of the microsomal marker in the mitochondrial fraction was 14% of the specific activity in pure microsomes, then 14% of the protein in the mitochondrial fraction was assumed to be microsomal. From the ratios of phospholipid to protein in pure microsomes and mitochondria, respectively, the percent of phospholipid in the mitochondrial fraction which is actually contributed by contaminating microsomes was calculated:

$$\%\ \text{microsomal lipid in mitochondrial fraction} = \frac{14\% \times 0.32}{(14\% \times 0.32) + (86\% \times 0.16)} = 25$$

where 0.32 is the lipid to protein ratio in pure microsomes and 0.16 is the lipid to protein ratio in pure mitochondria. These values from Colbeau et al. (1971) are for rat liver organelles. The values for microsomes and mitochondria from BHK cells are 0.72 and 0.39, respectively (Brotherus & Renkonen, 1977). The correct specific activity of the mitochondrial phospholipid was then derived from the apparent mitochondrial specific activity by subtracting the contribution of the contaminating microsomes.

$$\text{cor mitochondrial sp act.} = [\text{app mitochondrial sp act.} - 0.25(\text{microsomal sp act.})]/0.75$$

Assay of Phospholipid Transfer in Vitro. Phospholipid transfer activity was determined in a cell-free system by measuring the transfer of radiolabeled phospholipid from liposomes to mitochondria. Phosphatidylcholine liposomes (94 μg of phospholipid) containing a small portion of radiolabeled phospholipid and, in some cases, a trace of radiolabeled triolein were incubated with rat liver mitochondria (0.5–0.8 mg of protein) and a protein fraction in a total volume of 0.2 mL of STE buffer. The mitochondria had been previously heat treated as described by Bloj & Zilversmit (1977). Incubation was performed at 37 °C. The transfer was terminated by addition of 0.8 mL of ice-cold STE. The diluted reaction mixture was layered on 0.5 mL of 20% sucrose and centrifuged in an Eppendorf microcentrifuge, Model 3200 (Brinkmann), for 15 min at 4 °C. The mitochondrial pellet was suspended in 0.5 mL of H_2O and counted in 5.0 mL of Liquiscint. The percent of protein-specific phospholipid transfer was obtained by subtracting the amount of transfer found without the addition of phospholipid transfer proteins from the total amount of transfer. Apparent transfer due to fusion was monitored by the inclusion of the nontransferable marker [^{14}C]triolein either in the same liposomes with the labeled phospholipid or in liposomes with identical composition in a parallel assay.

Preparation of pH 5.1 Supernatant. The pH 5.1 supernatant fraction was prepared from rat liver as described by Bloj & Zilversmit (1977) except that rats had not been fasted, the homogenate was prepared in STE buffer, and the final supernatant, without a readjustment of pH, was dialyzed against 10 mM Tris-HCl and 10 mM β-mercaptoethanol, pH 7.2 (Tris–EtSH buffer), overnight at 4 °C. This fraction was then centrifuged at 15000 rpm for 15 min, 0–4 °C, to remove material which precipitated during dialysis.

A pH 5.1 supernatant fraction was prepared from BHK-21 cells by the same procedure as that used for rat liver except that the cells were homogenized with a Brinkmann Polytron as described above.

Partial Purification of Phosphatidylcholine Transfer Activity. Phosphatidylcholine phospholipid transfer protein from rat liver was purified by the procedure of Lumb et al. (1976) through the first Sephadex G-50 gel filtration step.

Phosphatidylcholine phospholipid transfer protein from BHK-21 cells was purified from cells (43 g wet weight; ca. 1.5×10^{10} cells) which had been stored at −70 °C. After the cells had thawed, a pH 5.1 supernatant fraction was prepared as described above. All subsequent steps were performed at 0–4 °C. The pH 5.1 supernatant was applied to a column (24 × 1.1 cm) of DEAE-cellulose (Whatman DE 52, microgranular). The column was washed with Tris–EtSH buffer, and the activity eluted with a linear gradient composed of 100 mL of Tris–EtSH buffer and 100 mL of 0.5 M NaCl in the same buffer. Fractions with phosphatidylcholine transfer activity were pooled and dialyzed overnight against Tris–EtSH buffer. The activity in the dialysate was precipitated by addition of ammonium sulfate to 95% saturation. The precipitate was collected by centrifugation at 15000 rpm for 15 min and then dissolved in 1.0 mL of Tris–EtSH buffer. This sample was applied to a column (55 × 1.1 cm) of Sephadex G-75. Fractions containing phosphatidylcholine transfer activity were pooled and served as the source of partially purified transfer protein. The preparation was stored unfrozen on ice.

Results

Purity of Subcellular Fractions. The activities of marker enzymes in microsomes and mitochondria from a typical fractionation of hepatocytes and BHK-21 cells are displayed in Table I. The degree of contamination of one subcellular fraction by the other was derived from these activities. The values for contamination of mitochondria by microsomes were used to correct the specific activity of mitochondrial phospholipid in cellular labeling studies as described under Experimental Procedures. The contamination of microsomes by mitochondria was not large enough to significantly alter the

Table I: Activity of Marker Enzymes

fraction	specific activity[a] NADPH-cyt *c* reductase	specific activity[a] succinate-cyt *c* reductase	contamination[b] (%)
hepatocyte			
microsomes	197 ± 16 (8)	2.42 ± 0.12 (4)	1.6
mitochondria	17.0 ± 1.2 (8)	147 ± 5 (4)	8.6
BHK-21			
microsomes	40.6 ± 2.5 (8)	1.23 ± 0.25 (7)	2.3
mitochondria	6.06 ± 0.56 (8)	85.6 ± 16.9 (7)	14.9

[a] Units: nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$. [b] Contamination of microsomes by mitochondria or of mitochondria by microsomes.

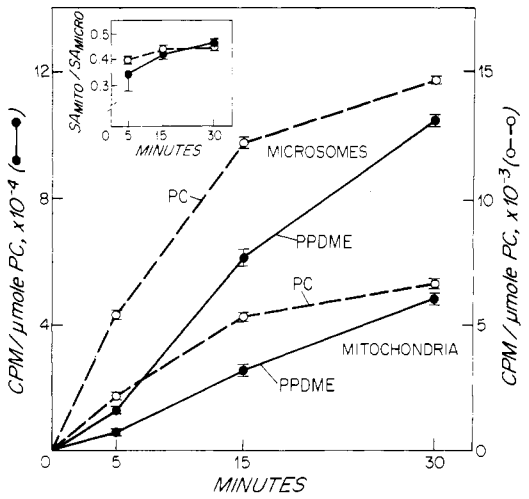

FIGURE 1: Labeling of hepatocyte phospholipid with [^{3}H]PDME and [^{14}C]choline. Cells were incubated with 0.16 μM [^{3}H]PDME and 2.9 μM [^{14}C]choline at 37 °C. Samples of the incubation mixture were removed periodically. Cells were homogenized, microsomes and mitochondria were isolated, and lipids were extracted from the subcellular fractions. The radioactivity and phosphorus contents were determined for each sample. Both quantities for the mitochondria were corrected for contamination of microsomes. The content of phosphatidylcholine was calculated from the phosphorus content and the phospholipid composition of the membranes. Values from two independent incubations and their averages are displayed.

specific activity of the microsomal phospholipid.

Incorporation of Radiolabeled Precursors into Phospholipids of Hepatocytes. Freshly isolated rat hepatocytes readily incorporated [*methyl*-^{14}C]choline and [1-^{3}H]PDME into phospholipid at 37 °C (Figure 1). The labeling of the mitochondrial phospholipid very rapidly followed that of the microsomes for both phospholipids over the 30-min incubation. Significant radioactivity was found in mitochondrial phosphatidylcholine and phosphatidyl-PDME after even very brief (5 min) incubation times. The ratios of specific activity of mitochondrial to microsomes for the two phospholipids (Figure 1, insert) are nearly identical for all incubation times, reflecting the similar rates of equilibration of the phosphatidylcholine and phosphatidyl-PDME between the two organelles. Similar patterns of labeling were observed when hepatocytes were incubated with [^{14}C]choline, [^{3}H]choline, or [^{3}H]PDME individually, at either low (0.15–3.0 μM) or high (1.0 mM) concentrations. Competition between choline and its propyl analogue, resulting in decreased incorporation of both precursors, was apparent when cells were labeled with both bases simultaneously.

The identity of the labeled phospholipids in the subcellular fractions was confirmed by thin-layer chromatography and comparison with phosphatidylcholine and phosphatidyl-PDME standards. More than 85% of the tritium count was associated with authentic phosphatidyl-PDME, and more than 95% of the ^{14}C ran with the phosphatidylcholine standard.

Table II: Labeling of Submitochondrial Fractions

	fraction OM[a]	fraction IM[b]
% total radioactivity[c]	92.6	7.4
% total phospholipid[c]	53.1	46.9
% total phosphatidylcholine[c]	57.5	42.5
specific activity[d]	1.61	0.17
relative specific activity[e]	9.47	1

[a] OM, outer membrane plus intermembrane space fraction. [b] IM, inner membrane plus matrix fraction. [c] % total amounts in mitochondria. [d] Relative to mitochondrial specific activity (=1.0). [e] Relative to IM specific activity (=1.0).

The submitochondrial location of the newly synthesized phosphatidyl[^{3}H]choline was investigated in hepatocytes by subfractionation of mitochondria following their isolation from cells incubated with [*methyl*-^{3}H]choline for 15 min. Two fractions were derived from the mitochondria: an outer membrane–intermembrane fraction (OM) and an inner membrane–matrix fraction (IM). Table II shows the distribution of radioactivity, phospholipid, and phosphatidylcholine between the two fractions. Values displayed are the averages of determinations from two independent experiments; the duplicates differed by less than 10%. Most of the newly made phosphatidylcholine is found in the OM fraction, while total phosphatidylcholine is distributed more equally between the two fractions. The specific activity of the newly synthesized phosphatidylcholine in the OM was 9.5 times that of the IM fraction.

The labeling patterns of microsomal and mitochondrial phosphatidylcholine in hepatocytes were examined further by labeling phosphatidylcholine via an alternate biosynthetic pathway: the successive methylation of phosphatidylethanolamine. Hepatocytes were first incubated with dimethylethanolamine; this treatment greatly increases the labeling of phosphatidylcholine from methionine (Sundler & Akesson, 1975). Phosphatidylcholine in microsomes and mitochondria is readily labeled by subsequent incubation with [*methyl*-^{14}C]methionine. As observed with [^{14}C]choline, the labeling of phosphatidylcholine in the mitochondrial compartment rapidly followed that of microsomal phosphatidylcholine. The ratio of specific activities of the two fractions was very similar to that observed with the [^{14}C]choline label.

The identity of labeled phospholipid was examined by thin-layer chromatography. More than 85% of the radioactivity in both the microsomes and mitochondria ran as authentic phosphatidylcholine at every time.

Hepatocytes incubated with [1-^{3}H]ethanolamine at 37 °C readily incorporated the radiolabel into phosphatidylethanolamine. In contrast to the pattern found for phosphatidylcholine the labeling of phosphatidylethanolamine in the mitochondria lags considerably behind that of the microsomes. This is reflected in the low ratios of mitochondrial specific activity to those of the microsomes. More than 95% of the radiolabel chromatographed as authentic phosphatidylethanolamine in analysis of phospholipid from subcellular fractions for all incubation times.

The movement of phosphatidylethanolamine from microsomes to mitochondria was further examined in hepatocytes by studying the labeling of subcellular phosphatidylethanolamine during incubation with unlabeled ethanolamine following a 15-min pulse of [1-^{3}H]ethanolamine (Figure 2). The

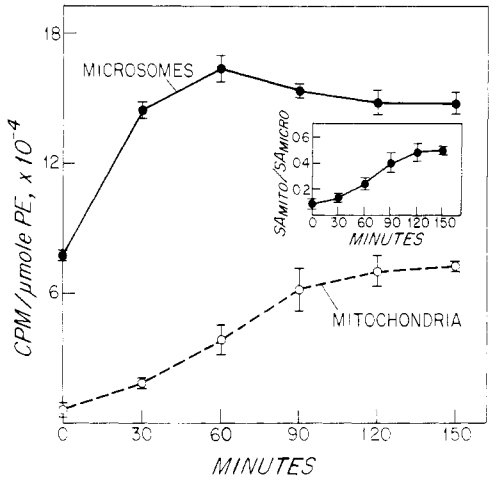

FIGURE 2: Chase with ethanolamine after labeling with [^{3}H]-ethanolamine. Cells were first incubated with 0.25 mM [1-^{3}H]-ethanolamine for 15 min at 37 °C. The incubation mixture was then diluted 4-fold with buffer containing 1.25 mM ethanolamine, and incubation was continued at 37 °C. Portions were periodically removed, and cells were isolated. Microsomes and mitochondria were isolated, and specific activities of the phosphatidylethanolamine were determined as described for Figure 1. Values from two independent incubations and their averages are displayed.

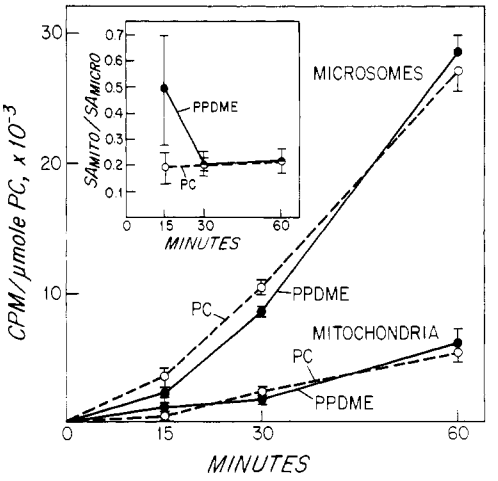

FIGURE 3: Labeling of BHK phospholipid with [^{3}H]PDME and [^{14}C]choline. Cells growing in monolayer were incubated with media containing 0.64 μM [^{3}H]PDME and 2.9 μM [^{14}C]choline at 37 °C. Separate, identical 60-mm dishes of cells were used for each time point. Following incubation, the cells were washed, scraped from the dishes, mixed with carrier, unlabeled cells, and homogenized. Microsomes and mitochondria were isolated, and phospholipids were extracted from the fractions. Specific activity of the fractional phospholipid was determined as described for Figure 1. Values from two independent incubations (separate dishes) and their averages are shown.

Table III: Effect of Temperature on Chase with Ethanolamine

temp (°C)	SA_{mito}/SA_{micro} at start of chase	SA_{mito}/SA_{micro} after 90-min chase
37	0.168	0.379
28	0.168	0.331
20	0.151	0.290
12	0.168	0.216

specific activity of the microsomal pool increased rapidly for 30 min, slowly for the next 30 min, and subsequently decreased slowly to a constant level at 120 min. The mitochondrial phosphatidylethanolamine specific activity increased at a fairly steady rate for 90 min and reached a constant level by 120 min. The ratio of mitochondrial to microsomal specific activities (Figure 2, insert) increased over 120 min from a low value to an apparently constant value of 0.5.

The effect of the temperature on the phosphatidylethanolamine movement during the chase is displayed in Table III. Cells were incubated for 15 min with [1-^{3}H]ethanolamine at 37 °C. They were then incubated in excess unlabeled ethanolamine at various temperatures. The increase in ratio of the specific activities of the two fractions over 90 min increased with temperature. At 0 °C there was no change in this ratio over at least 90 min.

Incorporation of Radiolabeled Precursors into Phospholipids of BHK Cells. As observed with rat hepatocytes, the phospholipids of microsomes and mitochondria in BHK cells are readily labeled by incubation of cells with [*methyl*-^{14}C]-choline and [1-^{3}H]PDME at 37 °C (Figure 3). The labeling of phospholipid in the mitochondria follows that of the microsomes, although this sequence of labeling is not as rapid as that found in hepatocytes. As in hepatocytes, however, the phospholipids labeled by the two different precursors equilibrate between the subcellular fractions at very similar rates (Figure 3, insert).

The identity of the labeled phospholipids in the subcellular fractions was confirmed by thin-layer chromatographic separation of the lipid extracts and comparison phospholipid standards. More than 90% of the ^{14}C and more than 90% of

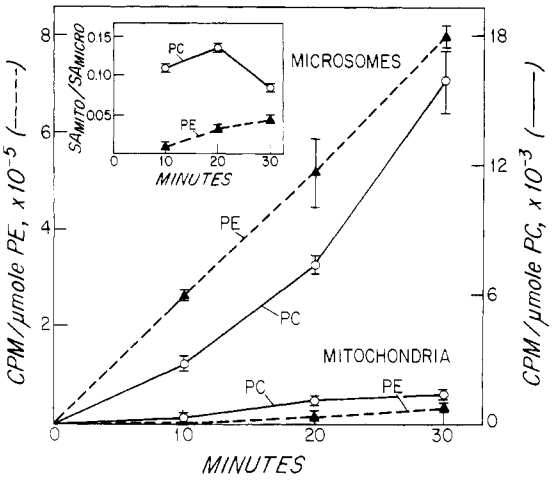

FIGURE 4: Labeling of BHK phospholipids with [^{3}H]ethanolamine and [^{14}C]choline. Cells growing in monolayer were incubated with media containing 0.9 μM [1-^{14}C]ethanolamine and 6 μM [^{14}C]choline at 37 °C. Other details are as described for Figure 3. Values for specific activities and their averages from two independent incubations are displayed.

the tritium count chromatographed as authentic phosphatidylcholine and phosphatidyl-PDME, respectively. This was found in both microsomes and mitochondria at all incubation times.

The pattern of phosphatidylethanolamine labeling similar to that found in hepatocytes was also detected upon incubation of BHK cells with [1-^{3}H]ethanolamine (Figure 4). These cells were simultaneously labeled with [^{14}C]choline which provided for a direct comparison between the movement of the two phospholipids. A substantial difference in the rates of labeling of the two subcellular compartments by the different precursors is demonstrated by the ratios of specific activities of the fractions (Figure 4, insert). Analysis of the phospholipids from the subcellular fractions revealed that at least 85% of the tritium and 90% of the ^{14}C chromatographed as authentic phosphatidylethanolamine and phosphatidylcholine, respectively.

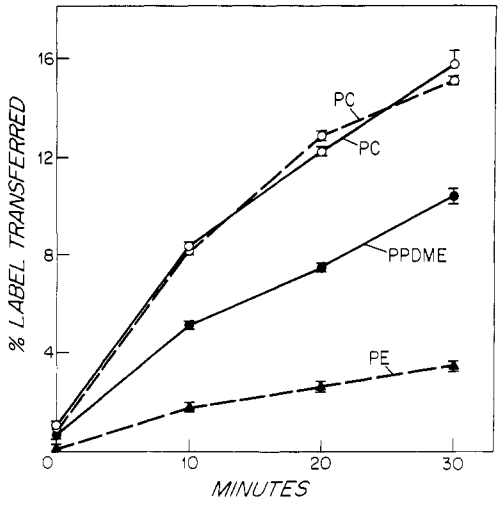

FIGURE 5: Transfer of phospholipid by pH 5.1 supernatant from rat liver. Liposomes (94 μg of phospholipid) containing phosphatidyl-[^{3}H]PDME and phosphatidyl[^{14}C]choline or phosphatidyl[^{3}H]-ethanolamine and phosphatidyl[^{14}C]choline were incubated with heat-treated, rat liver mitochondria (0.5 mg of protein) and pH 5.1 supernatant (0.1 mg of protein) from rat liver in a total volume of 0.2 mL of STE buffer at 37 °C. Following the incubation, mitochondria were isolated and the transferred radioactivity determined. Values for transfer of phospholipid in the absence of added pH 5.1 supernatant protein were subtracted from those for total transfer to yield the net transfer (displayed). Values from two independent incubations and their averages are shown.

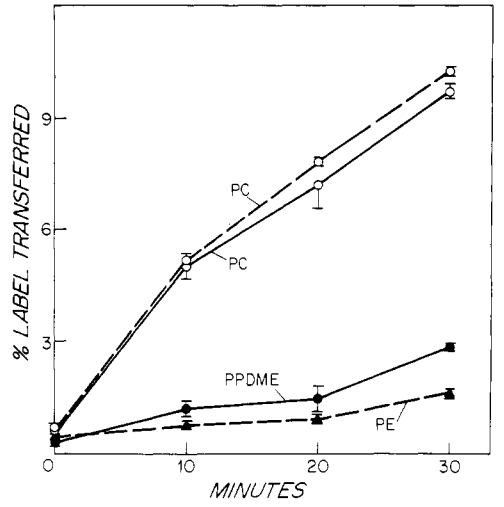

FIGURE 6: Transfer of phospholipid by pH 5.1 supernatant from BHK cells. Transfer of radiolabeled phospholipids from liposomes to mitochondria by pH 5.1 supernatant (0.15 mg of protein) from BHK-21 cells was measured as described for Figure 5. Values from two independent incubations are displayed.

Transfer of Phospholipid in Vitro. A pH 5.1 supernatant fraction from rat liver mediates the transfer of radiolabeled phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-PDME from liposomes to mitochondria (Figure 5). Phosphatidylcholine was transferred at a faster rate than the phosphatidylcholine analogue, which was transferred more rapidly than phosphatidylethanolamine. A pH 5.1 supernatant fraction from BHK cells also catalyzes phospholipid transfer (Figure 6); however, in contrast to the pattern of transfer observed for the liver fraction, phosphatidylethanolamine and phosphatidyl-PDME are both transferred at a rate much slower than that for phosphatidylcholine. In the absence of an added protein fraction, 1–4% of the liposomal phospholipid was recovered with the mitochondria. Net protein-dependent, phospholipid transfer was calculated by subtracting by subtracting the value observed in the absence of added protein.

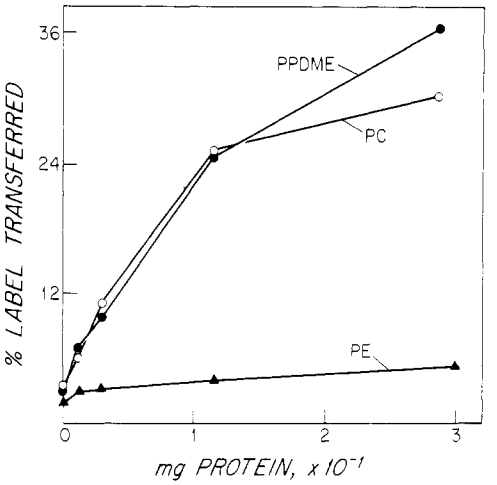

FIGURE 7: Transfer of phospholipid by partially purified phosphatidylcholine transfer protein from rat liver. Liposomes containing phosphatidyl[^{3}H]choline, phosphatidyl-[^{3}H]PDME, or phosphatidyl[^{3}H]ethanolamine and [^{14}C]triolein were incubated with rat liver mitochondria (0.8 mg of protein) and various amounts of phosphatidylcholine transfer protein for 15 min at 37 °C. Mitochondria were reisolated and transferred radioactivity measured. The fraction of total ^{3}H transferred was corrected for fusion or adsorption by subtracting the fraction of liposomal ^{14}C transferred.

The identity of transferred label was examined by extracting the phospholipids from mitochondria following an incubation with labeled liposomes and a protein fraction and then analyzing the phospholipids by thin-layer chromatography. More than 90% of the radioactivity chromatographed as the expected phospholipid (identical with that of the starting liposomes). The transferred phosphatidyl[^{14}C]choline and phosphatidyl-[^{3}H]PDME were further characterized by digesting the extracted mitochondrial phospholipid with phospholipase D and separating the hydrolyzed bases by thin-layer chromatography. More than 90% of the ^{14}C and 86% of the tritium ran as authentic choline and PDME, respectively.

Our standard assay for phospholipid transfer employed donor liposomes composed of phosphatidylcholine and containing small amounts of phosphatidyl[^{14}C]choline and either phosphatidyl-[^{3}H]PDME or phosphatidyl[^{3}H]ethanolamine. This approach facilitated the direct comparison of the transfer of the latter two phospholipids with the transfer of phosphatidylcholine. A similar pattern of specificity was revealed when transfer was examined by using phosphatidylcholine liposomes containing a small fraction of tritium-labeled phospholipid and the nontransferable marker [^{14}C]triolein. With this latter approach, the transfer in parallel assays, containing different labeled phospholipids, was compared. This assay corrects for possible fusion or adsorption which is induced by the added protein fractions.

For examination of the possibility that a substantial amount of phospholipid transfer activity was eliminated by the pH 5.1 fractionation, the transfer activity of high speed supernatant fractions prepared from cellular homogenates was examined. Similar patterns of phospholipid transfer to those observed by using the pH 5.1 supernatant fractions were found with these preparations.

Phosphatidylcholine-specific phospholipid transfer proteins were partially purified from rat liver and BHK-21 cells, and their ability to transfer phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-PDME was evaluated. Phosphatidylcholine transfer protein purified 45-fold from rat liver transfers phosphatidylcholine and phosphatidyl-PDME

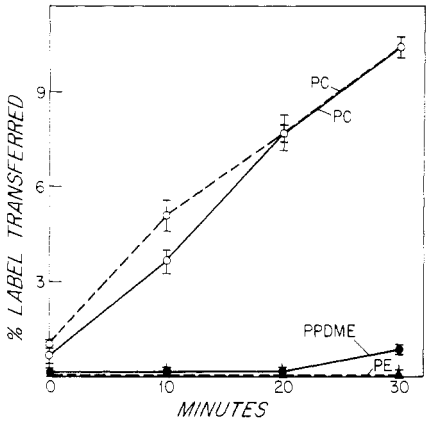

FIGURE 8: Transfer of phospholipid by partially purified phosphatidylcholine transfer protein from BHK cells. Transfer of phospholipid from liposomes to mitochondria (0.6 mg of protein) by 32 μg of partially purified transfer protein was determined as described for Figure 5. Values from two independent incubations and their averages are shown.

at identical rates but does not transfer phosphatidylethanolamine (Figure 7). A dramatically different pattern of phospholipid transfer is catalyzed by the phosphatidylcholine phospholipid transfer proteins purified 6-fold from BHK-21 cells (Figure 8). This preparation transfer phosphatidylcholine but shows no detectable activity toward the phosphatidylcholine analogue or phosphatidylethanolamine. This protein species accounted for more than 85% of the total phosphatidylcholine phospholipid transfer activity detected in the crude, soluble fraction.

Discussion

Sundler & Akesson (1975) have shown that incorporation of labeled bases into hepatocyte lipids occurs predominately via the CDP-ester pathway. They also found that [^{14}C]methyl of methionine could be incorporated into phosphatidylcholine by successive methylations of phosphatidylethanolamine. The final reactions of both these biosynthetic pathways occur in the endoplasmic reticulum in rat liver (Wilgram & Kennedy, 1963; Dawson, 1973; Bremer & Greenberg, 1961) and probably in most eukaryotic cells (Bell & Coleman, 1980). In BHK cells, choline incorporation into phosphatidylcholine also proceeds by the CDP-ester pathway (McMurray, 1975), and the enzyme catalyzing the final biosynthetic step in these cells is also microsomal.

A number of structural analogues of choline can be incorporated into phospholipid via the CDP-ester pathway (Akesson, 1977). This is the likely route for PDME incorporation into phospholipid, as further evidenced by the competition between choline and PDME for incorporation in both hepatocytes and BHK cells.

The present work reveals that the movement of phosphatidylcholine from microsomes to mitochondria is extremely rapid (Figures 1 and 3) in both hepatocytes and BHK cells. This transfer in hepatocytes is too fast to be studied under nonlabeling conditions (i.e., during a chase with unlabeled choline) since much of the equilibration would be complete by the time the chase could be initiated. The transfer of phosphatidyl-PDME from microsomes to mitochondria appears to occur at least as rapidly as the movement of phosphatidylcholine in both types of cells (Figures 1 and 3).

In striking contrast to the rapid movement of phosphatidylcholine and phosphatidyl-PDME, phosphatidylethanolamine is transferred at a slower rate both in hepatocytes and in BHK cells (Figures 2 and 4). In hepatocytes, the ratio of specific activities of the mitochondrial to microsomal phosphatidylethanolamine reaches a constant level (0.5) after approximately 120 min with a half-time of equilibration of about 70 min (Figure 2).

To evaluate the possible function of soluble phospholipid transfer proteins in the movement of phospholipids in intact cells, we have examined the pattern of specificity of (a) the total fraction soluble at pH 5.1 and (b) partially purified phosphatidylcholine transfer protein from both liver and BHK cells. Both types of preparation from rat liver transfer phosphatidylcholine and phosphatidyl-PDME more rapidly than phosphatidylethanolamine (Figures 5 and 7); this pattern is the same as that for the movement of these newly synthesized phospholipids from microsomes to mitochondria in hepatocytes.

In contrast, the pH 5.1 supernatant fraction from BHK cells transfers phosphatidylcholine rapidly and phosphatidyl-PDME and phosphatidylethanolamine only slowly (Figure 6). The partially purified phosphatidylcholine transfer protein (which accounts for most of the total transfer activity detected in the crude soluble fraction) does not transfer phosphatidyl-PDME at a detectable rate. The movement of phosphatidyl-PDME in vivo from microsomes to mitochondria in BHK cells cannot be distinguished from that of phosphatidylcholine. This result strongly suggests that a soluble transfer protein is not needed for rapid translocation in vivo. Such a conclusion involves the implicit assumption that the in vitro transfer assay measures the specificity of the proteins in vivo; this assumption is also necessary for the original postulation of such a function of these proteins in living cells. Previous studies (Wirtz et al., 1976a; Helmkamp, 1980; Demel et al., 1982) have indicated that the lipid composition of donor or acceptor particles in a cell-free system can influence the rate of transfer by phospholipid transfer proteins. None of these studies, however, revealed dramatic changes in the specificities of phospholipid transfer proteins by the altered assay conditions.

Kamp et al. (1977) have reported a high degree of specificity for phospholipid head groups by the phosphatidylcholine-specific phospholipid transfer protein from beef liver. They found no transfer activity toward phosphatidyl-PDME. In our hands, this same protein transferred the analogue at 60–70% of the rate for phosphatidylcholine. The reason for this discrepancy has not been determined.

It should be emphasized that, although the movement of phospholipids in the intact cells observed in our experiments does not reflect the specificity of soluble transfer proteins, it is nonetheless specific. In both cell types, the movement of phosphatidylcholine and its analogue, phosphatidyl-PDME, is very much faster than that of phosphatidylethanolamine in spite of the fact that the last step of biosynthesis of each case involves CDP-linked intermediates and is catalyzed by closely similar microsomal enzymes. This result excludes a simple mixing or fusion of the lipid components of the endoplasmic reticulum with mitochondria as the basis of the rapid labeling of the mitochondria.

Phospholipid transfer proteins may well play a role in intracellular phospholipid biosynthesis or metabolism in association with some other cellular components. Correlations between increased or specialized phospholipid metabolism and phospholipid transfer protein activity have been observed in the developing mouse lung (Engle et al., 1978), alveolar epithelial type II cells (Post et al., 1980), synaptosomes from rat brain (Wirtz et al., 1976b), and rat Morris hepatomas (Poorthuis et al., 1980). None of these studies provided substantive evidence for the direct role of phospholipid transfer

Table IV: Phospholipid Transfer Rates

phospholipid	time (min)	ratio[a]	fraction of lipid exchanged[b]
hepatocytes			
PC[c]	5	0.39	0.25
PC[d]	5	0.35	0.22
PPDME[e]	5	0.34	0.21
PE[f]	6	0.019	0.013
PE[g]			0.014
BHK-21			
PC[c]	5	0.17	0.11
PE[f]	7	0.0025	0.0014

[a] Specific activity of mitochondria/specific activity of microsomes. [b] Approximate fraction per minute of phospholipid in mitochondrial outer membrane derived from endoplasmic reticulum. [c] Phosphatidylcholine, [^{3}H]choline label. [d] Phosphatidylcholine, [^{14}C]methionine label. [e] Phosphatidylpropyldimethylethanolamine, [^{3}H]PDME label. [f] Phosphatidylethanolamine, [^{3}H]ethanolamine label. [g] Phosphatidylethanolamine, constant estimated from chase with unlabeled ethanolamine following [^{3}H]ethanolamine pulse.

proteins in intracellular phospholipid movement.

Although the rate of movement of newly synthesized phosphatidylcholine and phosphatidyl-PDME from microsomes to mitochondria was too rapid for kinetic analysis by the methods available, an approximate calculation can be made from the ratio of the specific activity of the mitochondrial lipid to the microsomal lipid after a very brief incubation with radiolabeled precursor. For example, in one of the experiments summarized in Table IV, the ratio after 5 min was 0.39, indicating that at least 39% of the mitochondrial phosphatidylcholine had been derived from the microsomes during that period, or 8%/min. The average specific activity of the microsomal pool during the period of transfer, however, was only half that attained at the end, if we assume that the labeling was linear during the 5-min period. The rate of exchange of the mitochondrial pool of phosphatidylcholine with the microsomal pool must therefore have been 16%/min. Finally, initially only phosphatidylcholine in the outer mitochondrial membrane participates in the exchange: since this portion constitutes only 0.6 of the total, it must be renewed at a rate of about 25%/min.

Table IV summarizes similar such calculations for several experiments with choline, PDME, and ethanolamine in hepatocytes and BHK cells. Since the cells contain no endogenous phosphatidyl-PDME, the content of phosphatidylcholine in microsomes and mitochondria, respectively, was used as the basis for determination of specific radioactivity in this case. For calculations of the rate for phosphatidylethanolamine transfer, 50% of the total mitochondrial phosphatidylethanolamine was assumed to be in the outer membrane.

The approximate nature of these calculations is recognized, but it is clear that phosphatidylcholine and phosphatidyl-PDME are translocated from microsomes to mitochondria at high rates, at least an order of magnitude more rapid than that of phosphatidylethanolamine. Furthermore, the value obtained from the above analysis for phosphatidylethanolamine transfer in hepatocytes is in close agreement with the rate observed in the experiment involving a chase with ethanolamine.

The necessity for such rapid transfer of phosphatidylcholine is not readily apparent. The rate of transfer in the rapidly growing BHK cell is at least 200-fold faster than would be necessary for a doubling of the mitochondrial phosphatidylcholine in the 16-h period of cellular doubling. In hepatocytes, the transfer rate is even faster than in BHK cells, while the doubling time has been estimated to be 160–400 days (Swick et al., 1956; McDonald, 1961). The rapid movement of phosphatidylcholine is also much faster than is necessary for the turnover of this phospholipid, a process with a half-time of 2–4 days in rat liver (McMurray & Dawson, 1969; Lee et al., 1973) and at least 15 h in tissue culture cells (Pasternak & Bergeron, 1970).

Our findings of very rapid movement of phosphatidylcholine in isolated hepatocytes are in contrast to those of Jungalwala & Dawson (1970) who observed a slower process with a half-time of the order of 15 min. In this early study of hepatocytes, however, rat liver was mechanically dispersed in a homogenizer with a loose-fitting plunger. It was necessary to supplement the cellular preparation so obtained with a supernatant fraction in order to obtain optimal lipid synthesis, indicating damage to the cells. Liver cells isolated after perfusion with collagenase, used in the present study, are close in appearance and function to normal hepatic parenchymal cells (Berry & Friend, 1969; Seglen, 1973).

The movement of phospholipids within the liver cell has also been studied in living rats (McMurray & Dawson, 1969; Stein & Stein, 1969; Blok et al., 1971) by injection of radioactive precursors and subsequent isolation of subcellular fractions from the liver. This approach is necessarily limited to an examination of a single time point for each rat, and by difficulties in manipulating the conditions of labeling and chase. After injection of [^{32}P]P_i, it was found that the specific activities of phospholipids were in the order microsomes > mitochondrial outer membrane > mitochondrial inner membrane. The results of our study are consistent with these findings as well as those of Eggens et al. (1979), who injected rats with [^{3}H]glycerol and found rapid labeling of microsomal phosphatidylcholine but only a slow rise (requiring several hours) in the specific activity of the mitochondrial inner membrane.

An interesting feature of the labeling of cellular phospholipids is the high, early labeling of mitochondrial phospholipid by PDME (Figure 3). A similarly elevated mitochondrial labeling at early incubation times was also observed when hepatocytes were incubated with high concentrations (1 mM) of choline or PDME. This initial, rapid incorporation may reflect some equilibration between the free choline (PDME) pool and the mitochondrial phosphatidylcholine. Sundler et al. (1972), in a study of the labeling of rat liver choline pools after injection of [^{14}C]choline, found that a portion of the injected label was incorporated into phosphatidylcholine by a pathway not involving CDP–choline. They postulated that this incorporation occurred by a base exchange reaction and further found that the rate of this exchange was only 5% of the rate of phosphatidylcholine synthesis via CDP–choline. The early mitochondrial labeling we observe may be due to this exchange reaction since the specific activities of the free choline and free PDME pools may be much higher than that of CDP–choline and CDP–PDME at the earliest time points. The high early labeling of mitochondria is not seen when phosphatidylcholine is labeled via methylation. This result suggests that the early labeling of the mitochondrial pool requires free choline.

The localization of most of the radiolabeled phospholipid in the mitochondrial outer membrane after a 15-min incubation with [^{3}H]choline (Table II) suggests that newly made phosphatidylcholine is initially transferred from the microsomes to the outer membrane and then to the inner membrane of the mitochondria. The ratio of mitochondrial to microsomal specific activities after 15 min was approximately 0.45 (Figure 1, insert). The ratios of the specific activities of the outer and

inner membranes to that of the total mitochondria are 1.6 and 0.17, respectively (Table II). From these ratios one can calculate the ratios of specific activities of mitochondrial outer and inner membrane to microsomes to be 0.72 and 0.077, respectively. Thus, the phosphatidylcholine in the outer membrane of the mitochondria is approaching equilibrium with that in the microsomes. Phosphatidylcholine of the mitochondrial inner membrane equilibrates with the outer membrane and, hence, with the microsomes at a much slower rate. The movement of labeled phospholipid from outer to inner membrane has been demonstrated in isolated mitochondria by Blok et al. (1971).

Although mitochondria were not subfractionated after labeling with [^{3}H]ethanolamine, the ratio of mitochondrial to microsomal specific activities for phosphatidylethanolamine at the apparent equilibrium level corresponds to the value predicted for equilibration of the phosphatidylethanolamine of the outer membrane with that of the microsomes, on the assumption that nearly all of the labeled mitochondrial phosphatidylethanolamine is in the outer membrane.

The data presented by this study are inconsistent with the postulate that phospholipid transfer proteins are the sole mediators of the transfer of phospholipids from endoplasmic reticulum to mitochondria. Minimally, our results suggest that the phosphatidylcholine phospholipid transfer protein from BHK cells, which accounts for at least 85% of the total phosphatidylcholine transfer activity in a crude, soluble fraction, is not necessary for the rapid transfer of phosphatidyl-PDME from microsomes to mitochondria in the intact cell. Our analysis is based on a comparison of transfer in vitro with phospholipid movement in vivo. The limitations of such an approach are recognized; the specificities and activities of transfer proteins in our cell-free system may not accurately reflect these properties of the transfer proteins in the living cell. A definitive evaluation of the role of phospholipid transfer proteins in phospholipid movement in vivo may require a genetic test involving mutants in which specific phospholipid exchange proteins are altered by mutations in their structural genes.

Acknowledgments

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References

Akesson, B. (1977) *Biochem. J. 168*, 401–408.

Akiyama, M., & Sakagami, T. (1969) *Biochim. Biophys. Acta 187*, 105–112.

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Bell, R. M., & Coleman, R. A. (1980) *Annu. Rev. Biochem. 49*, 459–487.

Berry, M. N., & Friend, D. S. (1969) *J. Cell Biol. 43*, 506–520.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Bloj, B., & Zilversmit, D. B. (1977) *J. Biol. Chem. 252*, 1613–1619.

Blok, M. C., Wirtz, K. W. A., & Scherphof, G. C. (1971) *Biochim. Biophys. Acta 233*, 61–75.

Borch, R. F., Bernstein, M. D., & Durst, H. D. (1971) *J. Am. Chem. Soc. 93*, 2897–2904.

Bremer, J., & Greenberg, D. M. (1961) *Biochim. Biophys. Acta 46*, 205–216.

Brotherus, J., & Renkonen, O. (1977) *Biochim. Biophys. Acta 486*, 243–253.

Chen, P. S., Jr., Birge, C. H., & Vagelos, P. R. (1969) *Anal. Chem. 28*, 1756–1758.

Colbeau, A., Nachbaur, J., & Vignais, P. M. (1971) *Biochim. Biophys. Acta 249*, 462–492.

Dawson, R. M. C. (1973) *Subcell. Biochem. 2*, 69–89.

Demel, R. A., Van Bergen, B. G. M., Van Den Eeden, A. L. G., Zborowski, J., & Defize, L. H. K. (1982) *Biochim. Biophys. Acta 710*, 264–270.

Eggens, I., Valtersoon, C., Dallner, G., & Ernster, L. (1979) *Biochem. Biophys. Res. Commun. 91*, 709–714.

Engle, M. J., van Golde, L. M. G., & Wirtz, K. W. A. (1978) *FEBS Lett. 86*, 277–281.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Greenawalt, J. W. (1974) *Methods Enzymol. 31*, 310–323.

Helmkamp, G. M. (1980) *Biochim. Biophys. Acta 595*, 222–234.

Jungalwala, F. B., & Dawson, R. M. C. (1970) *Biochem. J. 117*, 481–490.

Kamp, H. H., Wirtz, K. W. A., Baer, P. R., Slotboom, A. J., Rosenthal, A. F., Paltauf, F., & van Deenen, L. L. M. (1977) *Biochemistry 16*, 1310–1316.

Kates, M., & Sastry, P. S. (1969) *Methods Enzymol. 14*, 197–203.

Lee, T.-C., Stephens, N., Moehl, A., & Snyder, F. (1973) *Biochim. Biophys. Acta 291*, 86–92.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lumb, R. H., Kloosterman, A. D., Wirtz, K. W. A., & van Deenen, L. L. M. (1976) *Eur. J. Biochem. 69*, 15–22.

McDonald, R. A. (1961) *Arch. Intern. Med. 107*, 335–343.

McMurray, W. C. (1975) *Can. J. Biochem. 53*, 784–795.

McMurray, W. C., & Dawson, R. M. C. (1969) *Biochem. J. 112*, 91–108.

Pasternak, C. A., & Bergeron, J. J. M. (1970) *Biochem. J. 119*, 473–480.

Poorthuis, B. J. H. M., Van der Krift, T. P., Teerlink, T., Akeroyd, R., Hostetler, K. Y., & Wirtz, K. W. A. (1980) *Biochim. Biophys. Acta 600*, 376–386.

Post, M., Batenburg, J. J., Schuurmans, E. A. J. M., & van Golde, L. M. G. (1980) *Biochim. Biophys. Acta 620*, 317–321.

Seglen, P. O. (1973) *Exp. Cell Res. 82*, 391–398.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *J. Cell Biol. 32*, 415–438.

Spector, A. A., & Hoak, J. C. (1963) *Anal. Biochem. 32*, 297–302.

Stein, O., & Stein, Y. (1969) *J. Cell Biol. 40*, 461–483.

Sundler, R., & Akesson, B. (1975) *J. Biol. Chem. 250*, 3359–3367.

Sundler, R., Arvidson, G., & Akesson, B. (1972) *Biochim. Biophys. Acta 280*, 559–568.

Swick, R. W., Koch, A. L., & Hamda, D. T. (1956) *Arch. Biochem. Biophys. 63*, 226–242.

Wilgram, G. F., & Kennedy, E. P. (1963) *J. Biol. Chem. 238*, 2615–2619.

Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta 344*, 95–117.

Wirtz, K. W. A., & Zilversmit, D. B. (1968) *J. Biol. Chem. 243*, 3596–3602.

Wirtz, K. W. A., & Van Deenen, L. L. M. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, 49–50.
Wirtz, K. W. A., Geurts van Kessel, W. S. M., Kamp, H. H., & Demel, R. A. (1976a) *Eur. J. Biochem. 61*, 515–523.
Wirtz, K. W. A., Jolles, J., Westerman, J., & Neys, F. (1976b) *Nature (London) 260*, 354–355.
Wojtczak, L., Zaluska, H., Wroniszewska, A., & Wojtczak, A. B. (1972) *Acta Biochim. Pol. 19*, 227–234.
Wurtman, R. J., & Axelrod, J. (1963) *Biochem. Pharmacol. 12*, 1439–1441.
Yaffe, M. P., & Kennedy, E. P. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1279 (Abstr. No. 5895).

Preparation of Semisynthetic Insulin Analogues from Bis(*tert*-butyloxycarbonyl)-desoctapeptide-insulin Phenylhydrazide: Importance of the Aromatic Region B24–B26†

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ABSTRACT: Semisynthetic analogues of insulin were prepared from derivatives of desoctapeptide-(B23–30)-insulin (DOI). A1,B1-$(Boc)_2$-DOI (di-Boc-DOI) was converted to A1,B1-$(Boc)_2$-DOI-B22-phenylhydrazide (di-Boc-DOI-NHNH-C_6H_5) by the trypsin-catalyzed addition of phenylhydrazine in aqueous organic solvents at pH 6.5 [Canova-Davis, E., & Carpenter, F. H. (1981) *Biochemistry 20*, 7053–7058]. Treatment of di-Boc-DOI-NHNH-C_6H_5 with BNPS-skatole produced the phenyldiimide. The latter was coupled with a variety of protected peptides that, after removal of protecting groups, yielded the following compounds whose biological activities were compared to that of insulin in binding, in stimulation of hexose transport ( ), and in the stimulation of lipogenesis [ ], in terms of percent of insulin activity, all in the isolated epididymal fat cell: di-Boc-DOI 0.2, (0.1), [0.2]; di-Boc-DOI-NHNH-C_6H_5 0.5, (0.2), [0.5]; DOI 0.2, (0.2), [0.1]; DOI-$(Gly)^{B23}$ 0.2, (0.2), [0.1]; DOI-$(Gly\text{-}Phe)^{B23-24}$ 6.3, (6.3), [8.0]; DOI-$(Gly\text{-}Phe\text{-}Phe)^{B23-25}$ 17.0, (25.6), [24.7]; DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$ 59.0, (50.0), [69.0]. The semisynthetic derivatives represent a stepwise readdition of the aromatic residues near the C terminus of the B chain. A given analogue demonstrated comparable activity in all three biological assays. The results indicate that the stepwise addition of aromatic residues to the B-chain C terminus of DOI produces an increase in insulin-like activity. The biological activity of DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$, the derivative in which the aromatic region has been completely reassembled, is the same order of magnitude as that of insulin.

The trypsin-catalyzed removal of residues B23–30 of insulin results in the generation of desoctapeptide-insulin (DOI),[1] an insulin analogue possessing strikingly little capacity to elicit insulin-like effects (Young & Carpenter, 1961; Carpenter & Baum, 1962; Kikuchi et al., 1980). The highly aromatic region Phe^{B24}-Phe^{B25}-Tyr^{B26} has been postulated as important for the formation of insulin dimers (Blundell et al., 1972) and as critical for insulin receptor binding (Pullen et al., 1976). Removal of part of this region by the action of pepsin (Gattner, 1975) generates DOI-Gly-Phe-Phe, a derivative with 20% activity.

Several years ago, Ruttenberg (1972) proposed a method for the synthesis of human insulin from porcine insulin. The method suffered from serious deficiencies and has not been replicable (Gattner et al., 1978; Obermeier, 1978). Variations of Ruttenberg's proposal have been used in attempts to produce insulin analogues modified in the B24–26 aromatic region (Shanghai Insulin Research Group, 1973; Weitzel et al., 1976). The products of these attempts were poorly characterized both chemically and biologically, which leads one to question their validity.

Recently, a new method for the preparation of insulin analogues has been developed (Canova-Davis & Carpenter, 1978; Inouye et al., 1979; Canova-Davis & Carpenter, 1980, 1981). In it, trypsin was used to form a peptide bond between the carboxyl of Arg-22 of the B chain of DOI and an amino group, from either a peptide (Inouye et al., 1979) or some other molecule (Canova-Davis & Carpenter, 1978, 1980, 1981). The Japanese group has used their variation of this method, direct peptide coupling, in the preparation of human insulin from porcine DOI and synthetic human octapeptide B23–B30 (Inouye et al., 1979). The direct peptide coupling method was

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3′-bromoindolinine; BSA, bovine serum albumin; Bu^t, *tert*-butyl; DEAE, diethylaminoethyl; DOI, desoctapeptide-(B23–30)-insulin; DOI-Gly, DOI-$(Gly)^{B23}$; DOI-Gly-Phe, DOI-$(Gly\text{-}Phe)^{B23-24}$; DOI-Gly-Phe-Phe, DOI-$(Gly\text{-}Phe\text{-}Phe)^{B23-25}$; DOI-Gly-Phe-Phe-Tyr, DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$; di-Boc-DOI, A1,B1-$(Boc)_2$-DOI; di-Boc-DOI-NHNH-$C_6H_5$, A1,B1-$(Boc)_2$-DOI-B22-phenylhydrazide; DIEA, diisopropylethylamine; DMF, dimethylformamide; Et_3N, triethylamine; Et_2O, diethyl ether; F_3CCOOH, trifluoroacetic acid; HOAc, acetic acid; MeOH, methyl alcohol; Me_2SO, dimethyl sulfoxide; $NHNH_2$, hydrazide; OBu^t, *tert*-butyl ester; ONp, *p*-nitrophenyl ester; Z, benzyloxycarbonyl; THF, tetrahydrofuran; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; tri-Boc-insulin, A1,B1,B29-$(Boc)_3$-insulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hyroxymethyl)aminomethane; TLC, thin-layer chromatography. Unless otherwise noted all amino acids are of the L configuration.

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