Localization of phosphatidylethanolamine in microsomal membranes and regulation of its distribution by the fatty acid composition

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Abstract Rat liver microsomes were incubated with the monofunctional aminoreagent fluorescamine. Although the probe easily penetrated the membranes, two pools of phosphatidylethanolamine (PE) could be detected. The first pool rapidly reacted with the probe and comprised 80% of the total PE. The second pool exhibited a very slow interaction. The two pools showed differences in fatty acid composition as well as in their sites of attachment. In vivo labeling with ethanolamine, glycerol, and palmitic acid resulted in a higher specific activity in the first pool after 1 hr; equilibration with the second pool took about 3 hr. No equilibration between the pools could be detected under in vitro conditions. In vivo incorporation of labeled fatty acids showed that palmitic and stearic acids were mainly incorporated into phosphatidylethanolamine by de novo synthesis, while linoleic and arachidonic acids were introduced through deacylation-reacylation processes. Injection of liposomes consisting of labeled synthetic phosphatidylethanolamines into the portal vein was followed by uptake by the hepatocytes and incorporation of the lipids into the microsomal membranes. Depending on the fatty acid composition of the injected lipid, one of either of the two pools became labeled. ▇▇ It is suggested that the fatty acid composition of a given phospholipid molecule exerts a signal function directing the lipid to its final intramembranous location. — **Valtersson, C., L. Filipsson, and G. Dallner.** Localization of phosphatidylethanolamine in microsomal membranes and regulation of its distribution by the fatty acid composition. *J. Lipid Res.* 1986. **27**: 731–741.

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The arrangement of phospholipids in model and biological membranes can be studied by using biophysical and chemical approaches (1). Depending on the methods employed, answers may be obtained concerning structure, intramembrane distribution, relation to adjacent macromolecules, fluidity, and mobility (2, 3). The possibility of an intramembrane compartmentalization has been studied by applying a number of amino reacting chemical probes (4). The cross-linking reagent, 1,5-difluoro-2,4-dinitrobenzene, gave some interesting information concerning the microsomal membranes of the liver, defining possible types of compartmentalization and the relatively slow movement of labeled lipids between the various pools (5). The problems that may be investigated with this probe are, however, limited; the reaction with microsomal PE is relatively slow and, even at room temperature, requires hours. Consequently, other types of probes are necessary in order to obtain conditions close to an in vivo situation.

Fluorescamine (FLA) has been used for the study of PE in intact cells, even in inner mitochondrial membranes, and has been found to possess certain advantageous properties (6, 7). This probe is a monofunctional reagent that reacts with primary amines, and the reaction is very rapid even at 4°C (8). Its easy penetration, not only through model but also biological membranes, is an important property of this probe.

In this study we have applied FLA to study the distribution of PE in microsomal membranes in order to obtain information on the existence of possible compartments and the metabolic regulation of the lipids in these presumed compartments. The aim was to find out whether there exists a regulatory mechanism for lipid distribution in various pools, and whether the nature of the phospholipid molecule itself influences this arrangement.

MATERIALS AND METHODS

Chemicals

Fluorescamine was purchased from Sigma, goat anti-rat albumin IgG was from ICN, and protein-A Sepharose was

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from Pharmacia. Anilin (Merck) was redistilled before use. Unlabeled fatty acids and dipalmitoyl and dioleoyl phosphatidylcholine were obtained from Sigma. [1(3)-^{3}H]Glycerol (2.6 Ci/mmol), [1-^{3}H]ethanolamine (1 Ci/mmol), [1-^{14}C]palmitic acid (56 mCi/mmol), [1-^{14}C]stearic acid (56 mCi/mmol), [1-^{14}C]linoleic acid (56 mCi/mmol), [1-^{14}C]arachidonic acid (59 mCi/mmol), 1-palmitoyl,2-[1-^{14}C]linoleoyl-sn-3-glycerophosphoethanolamine (56 mCi/mmol), and 1-palmitoyl,2-[1-^{14}C]oleoyl-sn-3-glycerophosphocholine (57 mCi/mmol) were obtained from Amersham.

Synthesis of phospholipids

For synthesis of 1-palmitoyl,2-[^{14}C]palmitoyl-, 1-palmitoyl,2-[^{14}C]stearoyl-, and 1-oleoyl,2-[^{14}C]linoleoyl-phosphatidylethanolamine, the unlabeled PC with the corresponding fatty acid in sn-1 position was subjected to phospholipase A_2 to prepare lysophospholipids. N,N-carbonyldiimidazole derivatives of labeled fatty acids were prepared and together with the lyso-compounds were used for the synthesis of labeled PC, catalyzed by 4-dimethylaminopyridine (9). To convert PC to PE, the procedure of Smith, Montecucco, and Bennett (10) was used with the following modifications. The PC was dissolved in 1 ml of chloroform and 1 ml of 10% ethanolamine in 40 mM $CaCl_2$ was then added without adjustment of the pH. This solution also contained 40 units of phospholipase D from *Streptomyces chromofuscus* (Boehringer). The mixture was incubated at room temperature for 20 hr with continuous stirring. In the case of 1-palmitoyl, 2-[^{14}C]oleoyl-PE, the corresponding labeled PC was converted to PE according to the above procedure. The previously prepared lyso-compounds, the intermediate PC, and the final PE were purified with preparative HPLC. The column used was LiChrosorb Si60 (7 μm) from Merck, using the procedure of Geurts Van Kessel et al. (11).

In vivo labeling

In vivo labeling was always performed through injection into the portal vein of rats under Nembutal anesthesia. [^{3}H]Glycerol (80 nmol, 200 μCi) and [^{3}H]ethanolamine (80 nmol, 80 μCi) in 0.9% NaCl were injected into the portal vein. [^{14}C]Palmitic acid (30 nmol, 1.7 μCi), [^{14}C]stearic acid (30 nmol, 1.7 μCi), [^{14}C]linoleic acid (148 nmol, 8.3 μCi), and [^{14}C]arachidonic acid (56 nmol, 3.2 μCi) in toluene were supplemented with 5 mg of dry fatty acid-free bovine serum albumin (Sigma), evaporated to dryness, and finally dissolved in 0.9% NaCl for the portal injection. In the case of labeled phospholipids, 0.1 μCi was mixed with egg phosphatidylcholine (Lipid Products, South Nutfield, England) in a molar ratio of 1:2 in chloroform. After evaporation of the solvent, the mixture was vortexed in 0.9% NaCl and sonicated until it was clear (5 min). The sonicated mixture was centrifuged at

2000 g for 20 min and the supernatant was used for in vivo labeling.

Preparation of microsomes and model membranes

Overnight fasted rats were used for the preparation of total, rough, and smooth microsomes from liver (12). The microsomal fraction and subfractions were resuspended in 100 mM NaCl containing 50 mM phosphate buffer, pH 7.8, and pelleted by centrifugation at 105,000 g for 60 min. A lipid extract from microsomes was used for preparation of model membranes. Total microsomes were extracted with chloroform–methanol–water 2:1:0.2 and the mixture was partitioned twice with water. Portions of the chloroform phase were evaporated under a stream of nitrogen. The lipid films in the glass tubes were placed under high vacuum for 1 hr in a freeze-dryer to remove traces of solvent. The lipids were subsequently treated with 50 mM phosphate buffer, pH 7.8, and dispersed by extensive vortexing at room temperature.

To determine contamination in isolated rough and smooth microsomes, various membrane fractions of liver homogenates from fasted rats were prepared. Golgi vesicles (13), plasma membranes (14), and lysosomes (15) were isolated using established procedures and were used for determination of specific marker enzymes. The values obtained were: for CMP-NANA transferase (Golgi vesicles) 929 pmol of NANA transferred per 10 min per mg of protein; for AMPase (plasma membranes) 0.84 μmol of P_i per min per mg of protein; and for acid phosphatase (lysosomes) 1.14 μmol of P_i per min per mg of protein. These specific activities were used to calculate the percentage contamination (on a protein basis) in the isolated rough and smooth microsomes. The various enzyme activities were determined using previously described procedures (6, 16, 17).

Fluorescamine treatment

In order to label PE in the microsomal membranes, 5 mg of microsomal protein in 100 mM NaCl and 50 mM phosphate buffer, pH 7.8, in a final volume of 1 ml was mixed with 100 μl of 50 mM FLA in acetone (if not otherwise indicated) while vortexing. After 15 sec of incubation at 20°C, 100 μl of 250 mM aniline was added in acetone. In a series of control experiments, aniline was found to be most effective in quenching all nonreacted FLA within the hydrophobic region of the membrane. The mixture was used for lipid extraction and phospholipid analyses. In experiments with model membranes, the multilamellar vesicles (0.6 mg of total lipid) were treated with FLA as described above for microsomes.

Lipid extraction and separation

For extraction of lipids, 1 ml of incubation mixture was supplemented with 5 ml of methanol followed by addition

of 10 ml of chloroform. Extraction was performed at room temperature for 2 hr under an N_2 atmosphere. At the end of incubation, the suspension was partitioned with 5 ml of water; this step was repeated. The chloroform phase was dried and the lipids were dissolved in a small volume of chloroform. TLC was performed on silica gel 60 plates (Merck) developed in chloroform–methanol–acetic acid–water 65:25:2:3 (v/v). In this system, PE (R_f = 0.7) was well separated from the PE–FLA derivative (R_f = 0.85).

Lipid–phosphorus

After chromatography, the plate was sprayed with ninhydrin to make PE visible. This spot and the PEr spot, which had a yellow color, were scraped off and hydrolyzed overnight by heating in a sand bath at 150°C with 1 ml of perchloric acid. Samples were placed in an oven at 300°C for evaporation. The contents of the tubes were subsequently treated with 50 μl of 5 N H_2SO_4 and 0.2 ml of H_2O, and the samples were placed in a boiling water bath for 10 min in order to hydrolyze pyrophosphates. This was followed by addition of 0.5 ml of water, 50 μl of 2.5% ammonium molybdate, and 50 μl of 10% ascorbic acid. The sample was finally heated at 45°C for 20 min, and the absorbance was measured at 820 nm.

For measurements of radioactivity, the PE and PEr spots were scraped into screw-capped scintillation vials; 1 ml of methanol was added and the vials were heated to 50°C for 1 hr. Radioactivity was measured in a scintillation counter after addition of 10 ml of Aqualuma Plus.

The tables and the figures of this report give the mean values from six to nine experiments.

Analysis of fatty acids

The spots were scraped into 3 ml of 3 M HCl in methanol for hydrolysis and methylation (18). The tubes were sealed in an N_2 atmosphere and placed in an oven at 80°C for 2 hr. The methylated fatty acids were extracted with 1 ml of hexane, and the extracts were washed first with 0.1% Na_2CO_3 and then with water. Samples of the washed extract were analyzed by capillary gas–liquid chromatography with cyanosiloxane 60 as stationary phase (19).

The fatty acid compositions of the total PE fraction, the PEunr and PEr fractions, isolated by TLC, were determined. In order to analyze the fatty acids at individual positions of the lipid, PE was treated with phospholipase A_2. This enzyme was purified from *Naja naja* venom (Sigma), as described by Deems and Dennis (20). For the hydrolysis, the PE samples were mixed with egg PC, and small unilamellar vesicles were prepared by sonication. The treatment with phospholipase A_2 was performed at 37°C for 30 min in the presence of 1 mM $CaCl_2$. The lyso-PE was separated by TLC, and the fatty acid composition was determined as described above. In this way it

was possible to obtain the fatty acid composition at the sn-1 position of both total PE and PEunr fractions. The difference between total PE or total PEunr and their corresponding lyso-compounds gave the fatty acid composition at the sn-2 position. Since PEr is not hydrolyzed by phospholipase A_2, the fatty acid distribution in the lysolipid was calculated from the data of total lyso-PE (before FLA treatment), lyso-PEunr, and PEr.

RESULTS

Reaction with microsomes

In order to study the interaction of PE with FLA, the microsomes were incubated in the presence of increasing concentrations of the probe. The reaction of FLA with PE is very fast (100–500 msec), and the excess of unreacted probe was quenched with aniline after 15 sec. After the incubation, the lipids were extracted, and the PE that had reacted with FLA was separated by TLC from the fraction that had not. When the unreacted PE, expressed as percent of the total phosphatidylethanolamine, was plotted in a semilogarithmic scale, two pools of PE were apparent (**Fig. 1A**). Already at low reagent concentration, a rapid interaction was observed with 80% of PE. The remaining PE, making up about 20% of the total, was characterized by a very slow interaction with FLA even at high reagent concentrations. When the incubations were performed in the presence of 0.25% Triton X-100, the membrane barrier was completely abolished. At the same time, the second pool was eliminated and all PE rapidly reacted with the probe. Fig. 1A also shows the result of incubation in the presence of 0.05% Triton. At this detergent concentration, the permeability barrier for macromolecules is eliminated without disrupting the membrane structure (21, 22). Under these conditions the second pool is not removed, suggesting that the presence of the slow-reacting pool of PE in the microsomal membranes may be explained as a structural property of the membrane itself and not simply caused by the permeability barrier. In order to investigate whether microsomes are permeable to the probe or not, the interaction of intramicrosomal albumin with FLA was studied. Microsomes were incubated with increasing amounts of FLA for 15 sec in the absence of detergent. After quenching with aniline, the microsomes were solubilized with detergent, and anti-rat albumin IgG was added. The immunoprecipitation was followed by separation of the antigen–antibody complex on protein A-Sepharose. After dissociation of the complex and separation of albumin on a slab gel, the fluorescence of the isolated protein was measured. Relatively high labeling of albumin was detected at very low FLA concentrations. These experiments again demonstrate that the probe easily penetrates the microsomal membrane. When intact
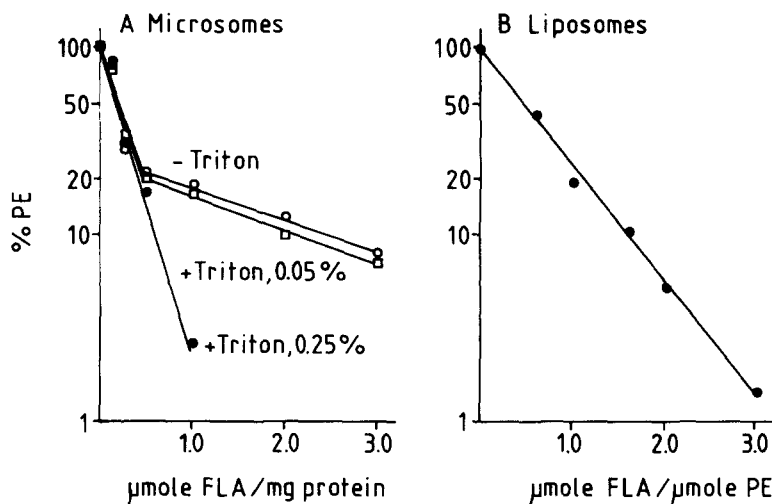
Fig. 1. Interaction of FLA with PE. A, Microsomes were incubated with various concentrations of FLA in the absence and in the presence of 0.05% and 0.25% Triton X-100. The percent of unlabeled PE was determined. B, Liposomes were prepared from the lipid extract of microsomes and treated with various concentrations of FLA.

microsomes in the absence of detergent were incubated with anti-rat albumin IgG, no precipitation was obtained, indicating the intraluminal localization of all microsomal albumin.

In order to further analyze interaction of the probe with PE, the total microsomal lipids were extracted and multilamellar vesicles were prepared. Incubation with increasing concentrations of FLA demonstrated the presence of a single PE pool which rapidly reacts with the probe (Fig. 1B). The rapid interaction of all PE with the multilamellar structure shows that FLA readily penetrates bilayer structures, and also that the slowly reacting pool that was found in the microsomes is not elicited only by the phospholipid composition.

The total microsomal fraction is known to be contaminated with Golgi and plasma membranes and lysosomes

(23). If the PE in these membranes is organized differently than in the microsomes, the PEunr pool could be explained by the presence of contaminating membranes and not by a separate pool in the microsomes. To analyze this problem we have prepared rough and smooth microsomes, since the latter contain twice as much lysosomal and plasma membrane, and, furthermore, 10 times more Golgi membrane contamination (24–26). It is inferred from the data in **Table 1** that approximately 10% of protein in the rough microsomes and 20% of protein in smooth microsomes belong to contaminating Golgi vesicles, plasma membranes, and lysosomes. Experiments with FLA demonstrated that the amounts of the two pools, described above for total microsomes, are practically identical in the two subfractions. For rough microsomes, the pool that reacted made up 79% of the total, while this value was

TABLE 1. Distribution of some marker enzymes in rough and smooth microsomes[a]

Marker Enzymes	Rough Microsomes		Smooth Microsomes	
	Specific Activity	Calculated Contamination	Specific Activity	Calculated Contamination
		% of total		
NADPH-cytochrome c reductase[b]	0.039		0.037	
Glucose-6-phosphatase[c]	0.36		0.35	
CMP-NANA transferase[d]	4.1	0.4	37.5	4.0
AMPase[c]	0.03	3.6	0.06	7.2
Acid phosphatase[c]	0.07	6.1	0.13	11.4

[a] Enzyme activities were measured in Golgi vesicles, plasma membranes, and lysosomes isolated from liver of starved rats. These values were used to calculate contamination in the microsomal subfractions.
[b] Micromoles of NADPH oxidized per min per mg protein.
[c] Micromoles P$_i$ per min per mg protein.
[d] Picomoles of NANA transferred per 10 min per mg protein.

81% for smooth microsomes. Therefore, the presence of two pools in microsomes cannot be explained by contamination with other intracellular organelles.

In vivo labeling with [³H]glycerol and [³H]ethanolamine

In vivo labeling with radioactive glycerol is an established procedure for following the de novo biosynthetic pathway of phospholipids that are not influenced by other factors, such as base exchange or deacylation–reacylation processes. The radioactivity was distributed in a characteristic manner in the two PE compartments of the microsomes when in vivo labeling with [³H]glycerol was performed (**Fig. 2A**). Compartmentalization was obtained by applying 1 μmol of FLA per mg of microsomal protein; in this way the PE became distributed into the reacted (PEr) and the unreacted (PEunr) compartments, comprising 80% and 20% of the total PE, respectively. During the first 30 min, the specific radioactivity was much higher in the reacted portion; equilibration between the two compartments took 3 hr. A generally similar picture was obtained when [³H]ethanolamine was injected as label (Fig. 2B). These experiments indicate that the newly synthesized PE is associated with that part of the lipid which easily reacted with the probe. The exchange process with molecules of the nonreacted compartment is a relatively slow process.

In vitro incubation of labeled microsomes

It was deemed important to investigate whether or not the exchange of the labeled PE between the two compartments, described above, was an in vivo phenomenon, not occurring under in vitro conditions. For this reason, liver microsomes were prepared from rats labeled with [³H]glycerol 15 min prior to decapitation, and the membranes were incubated in vitro at 37°C. Samples were removed at predetermined times; they were treated with FLA, and the two PE compartments were isolated (**Fig. 3**). In agreement with results of previous experiments (see Fig. 2A), the specific radioactivity in the PEr pool after the 15-min in vivo labeling was considerably higher than in the PEunr pool. In vitro incubation of the microsomes up to 180 min at 37°C did not change the distribution of labeled PE, judging from the analysis of the two compartments obtained after FLA treatment of the microsomes. Thus, the experiment demonstrates that movement of the lipids from one compartment to the other, as observed under in vivo conditions, represents an active process not attained as a passive translocation during in vitro conditions.

In vivo labeling with [¹⁴C]fatty acids

The compartmentalization was most obvious when the in vivo incorporation of labeled fatty acids was explored. In these experiments, albumin-bound fatty acids were injected into the portal vein of rats; the fatty acids were then taken up by the hepatocytes and became incorporated into microsomal PE (27). No change in the normal fatty acid composition due to the incorporation into PE could be detected (data not shown). The distribution of labeled palmitic acid was quite dramatic within the first 60 min (**Fig. 4A**). The specific activity in PEr exceeded by 10 times that of the PEunr, and there was a slow rate of increase during the next 2 hr in the latter compartment. The incorporation pattern was very similar when [¹⁴C]stearic acid was administered (Fig. 4B). On the other hand, both linoleic and arachidonic acid displayed a more equal and
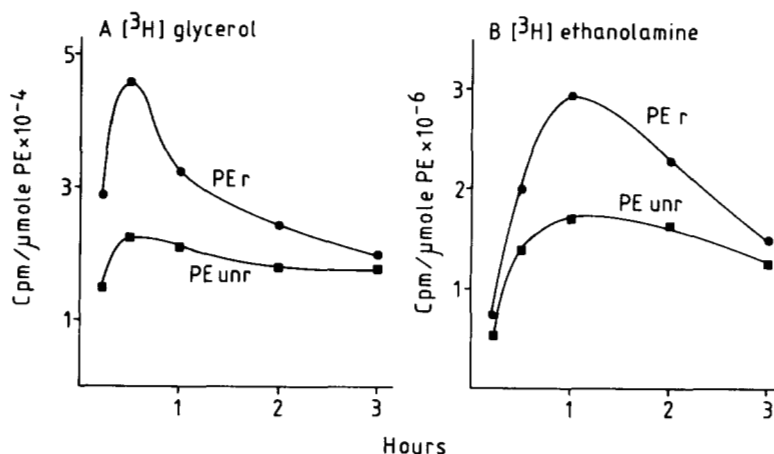

Fig. 2. Appearance of in vivo injected [³H]glycerol and [³H]ethanolamine in microsomal PE compartments. [³H]Glycerol (A) or [³H]ethanolamine (B) was injected in the portal vein and at various time-points the microsomes were isolated and treated with 1 mM FLA/mg protein. The specific radioactivity in the reacted PE (PEr) and in the unreacted PE (PEunr) is plotted as a function of in vivo incorporation time.
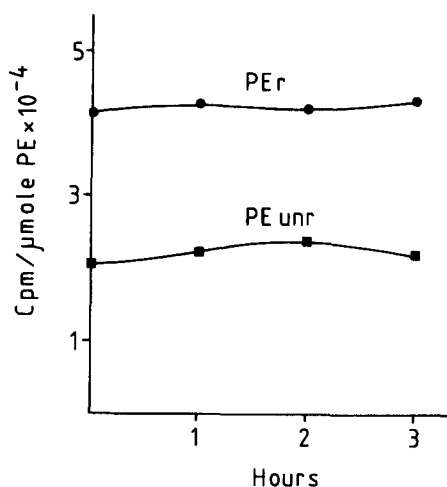
Fig. 3. In vitro incubation of in vivo labeled microsomes. Rats were labeled in vivo with [³H]glycerol and after 30 min microsomes were isolated and incubated at 37°C. At various time-points, FLA treatment was performed and the specific radioactivity was determined in the PEr and PEunr compartments.

parallel incorporation into the PEunr pool compared with PEr (Figs. 4C and D).

Some of the fatty acids are mainly incorporated into phospholipids through the process of biosynthesis, and others through exchange reactions (28). For this reason, the molar uptake of the individually labeled fatty acids was calculated in relation to the uptake of glycerol (**Table 2**). This ratio was 1 when equal moles of radioactive precursors and glycerol were incorporated. A greater ratio than 1 can be explained by the notion that a precursor is incorporated by exchange reactions rather than by de novo synthesis. The quotient for the fatty acids was calculated from the natural molar relationship of the individual fatty acids compared to glycerol. The ratio of ethanolamine incorporation per glycerol unit increased to 3.8 in PEunr and 2.5 in PEr after 3 hr; this implies that there was probably a considerable exchange. Palmitic and stearic acids were incorporated into the PEr pool with a ratio of 1–2. On the other hand, the PEunr pool had a considerably lower ratio. Linoleic and, particularly, arachidonic acid were both characterized by an extensive uptake compared to glycerol. For linoleic acid, the molar incorporation was 3 times higher in the PEr as compared to the PEunr compartment. Arachidonic acid had a very high turnover rate in both compartments, particularly in the PEunr where the rate was about twice as high as in the PEr pool.

The results of the above experiments suggest that palmitic and stearic acids do become incorporated into the PE molecule in vivo mainly as a result of de novo synthesis. Linoleic and arachidonic acids are obviously participating extensively in deacylation–reacylation reactions.

Fatty acid composition

The percentage distribution of the different fatty acids in the two PE pools is shown in **Table 3.** Some very striking differences in fatty acid composition between the two pools are apparent. Evidently, 15:0, 18:2, and 20:3 were all enriched in the PEunr pool, while the percentage distribution of 20:4 and 22:6 was about 2 times higher in the PEr compared to the PEunr compartment. By using phospholipase A_2, we have also investigated the fatty acid distribution at the sn-1 and sn-2 positions of the PE molecules. The two main saturated fatty acids, 16:0 and 18:0, were found only at the sn-1 position in PEr, while in the PEunr pool they were also present at the sn-2 position. We also found that 18:1 was located only at the sn-1 position in PEr, while it was present mainly at the sn-2 position in the other pool. Linoleic acid (18:2) appeared only at the sn-2 position of PEr and at both positions in PEunr where its total amount exceeded 4 times that of the former pool.

In vivo uptake of phospholipids by the endoplasmic reticulum

One experimental approach to test the specificity of phospholipid distribution is to study the incorporation of
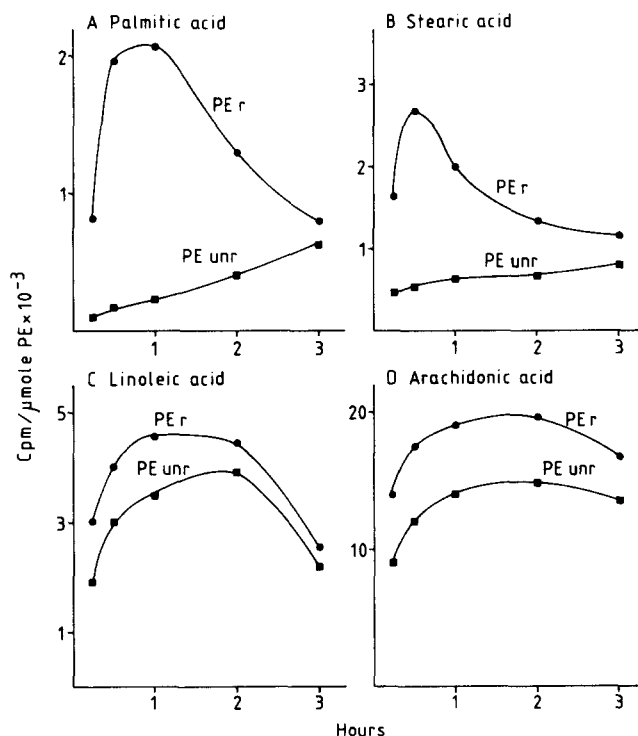

Fig. 4. Time-course of incorporation of in vivo injected fatty acids in the two PE compartments. [¹⁴C]Palmitic acid (A), [¹⁴C]stearic acid (B), [¹⁴C]linoleic acid (C), and [¹⁴C]arachidonic acid (D) were injected into the portal vein in albumin-bound form and at various time-points microsomes were isolated and treated with FLA.

TABLE 2. Ratio of ethanolamine and fatty acid incorporation to glycerol incorporation into microsomal PE[a]

Time of Incorporation	[³H]Ethanolamine		[¹⁴C]16:0		[¹⁴C]18:0		[¹⁴C]18:2		[¹⁴C]20:4	
	PEr	PEunr	PEr	PEunr	PEr	PEunr	PEr	PEunr	PEr	PEunr
15	1.2	1.6	1.1	0.2	1.7	0.9	11.8	3.8	10.2	22.4
30	1.3	1.9	1.6	0.3	1.7	0.6	9.6	4.4	7.7	20.4
60	2.5	2.5	2.4	0.3	1.7	0.8	14.9	5.2	11.9	24.9
120	2.3	2.6	1.9	0.5	1.5	1.0	19.2	6.7	16.2	30.7
180	2.5	3.8	1.5	0.8	1.4	1.3	14.7	4.3	18.2	31.5

[a] The specific activity of PE after in vivo [³H]glycerol labeling was taken as 1. The molar ratio of incorporation of [³H]ethanolamine and the various fatty acids to [³H]glycerol was calculated.

lipids into hepatocytes. After in vivo incorporation of [³H]glycerol, microsomal PE and PC were isolated, and lipid mixtures containing either of these lipids were sonicated to obtain liposomes that were injected into the portal vein of rats. The labeled lipids were taken up by the hepatocytes and appeared in the microsomal membranes as constitutive components. The uptake of labeled microsomal PE into liver microsomes was followed (data not shown). A maximal incorporation was reached after 60 min, followed by a slow decrease. During this time, label could be detected only in PE. When sonicated, labeled microsomal PC liposomes were injected into the portal vein, the incorporation pattern was very similar to that obtained with PE. As expected, the injected radioactivity was present in PC and not in any other phospholipid. These experiments show that the labeled glycerol moiety of the injected phospholipid is taken up by the hepatocyte and incorporated in the endoplasmic reticulum as an intact phospholipid and not as free glycerol, which would have been the case if the lipid were subjected to intracellular degradation.

In the following experiments, the individual species of PE with different fatty acid composition were injected into the portal vein and the distribution of the specific PE was followed. It is important in these types of experiments that the injected phospholipid to a large extent remains intact and unmetabolized. Under in vivo conditions, there are possibilities for modification of PE, such as involvement in transacylation processes or methylation into PC. **Table 4** shows the distribution of PE having different types of fatty acids. In all these PE forms, the fatty acid at the *sn*-2 position was labeled and the distribution was followed by determination of the radioactivity in isolated individual lipids. The uptake was quite different when microsomes and mitochondria were compared. The specific activities in microsomes exceeded by 3 or 4 times the specific activities in the mitochondria. Three of the injected lipids remained as PE both in microsomes and mitochondria, and only a small portion, about 10%, was transformed into PC. Dipalmitoyl PE exhibited a somewhat different pattern since a large portion of the radioactivity appeared in PC after 1 hr. No sizeable amount of radioactivity was

TABLE 3. Distribution of fatty acids in PE-reacted and unreacted compartments[a]

Fatty Acid	PE-Reacted			PE-Unreacted		
	Total	Pos. 1	Pos. 2	Total	Pos. 1	Pos. 2
			% of total			
15:0	0.7	1.0	0.4	3.1	4.0	2.3
16:0	18.5	36.0	1.0	22.9	34.0	11.8
17:0	0.5	0.2	0.8	0.7	0.2	1.2
17:1	0.9		1.8	1.9	3.9	
18:0	25.1	49.6	0.6	26.0	42.5	9.5
18:1	5.7	11.5		7.7	1.5	14.0
18:2	3.6		7.2	14.0	10.0	18.0
20:3	0.5	1.10		3.5	5.1	1.9
20:4	22.1		44.2	12.3		24.6
20:5	0.8		1.6	0.1		0.2
22:5	1.7		3.4	0.5		1.0
22:6	19.8		39.7	7.5		15.0

[a] After incubation with FLA, PE pools were separated and, after hydrolysis and methylation, the total fatty acid content was determined by capillary gas–liquid chromatography. The same lipids were also treated with phospholipase A₂ and the fatty acid content of the lyso-compound was analyzed.

TABLE 4. Uptake of injected PE into microsomes and mitochondria in vivo[a]

Injected	Microsomes		Mitochondria	
	PE	PC	PE	PC
	cpm/μmol × 10⁻³			
16:0/16:0	30.7	12.9	10.3	6.4
16:0/18:1	117.0	11.7	51.8	4.9
16:0/18:2	125.8	12.5	37.8	4.5
18:1/18:2	35.3	4.2	7.12	1.1

[a] The different PE species were injected into rats and after 60 min the specific radioactivities were determined in the PE and PC of microsomes and mitochondria.

found in other phospholipids. These experiments demonstrate that the intraportal injection of PE is a suitable method for studying PE distribution in the hepatocytes; in most cases the extent of modification reactions, such as transacylation and methylation, remained at a low level during the incorporation period and under the conditions employed.

As described above, the microsomal PE can be divided into two compartments on the basis of its reactivity with fluorescamine. Various PE molecules were injected into the portal vein; they were taken up by the hepatocytes and became incorporated into the microsomal membranes. The distribution of these lipids in the two FLA compartments was studied by determining the radioactivity after reaction of the isolated membranes with the probe. In the initial phase, dipalmitoyl PE was found mainly in the compartment that did not react with FLA; the difference in specific activity between the two pools after 1 hr was

8-fold (**Fig. 5A**). The incorporation experiment with 16:0/18:0-PE gave similar results: large differences in specific radioactivity between the two compartments, with low remaining radioactivity after 2 hr (Fig. 5B). By contrast, 16:0/18:2-PE exhibited an identical incorporation pattern between the two pools during the investigation (Fig. 5C). Similar experiments were also performed with 16:0/18:1-PE and 18:1/18:2-PE. The distribution of these lipids, together with the others described above after 1 hr of incorporation, is presented in **Table 5.** Three of the PE forms (16:0/16:0-, 16:0/18:0-, and 16:0/18:1-PE) showed 8-, 7-, and 2-fold increases in specific activities, respectively, in the PEunr pool compared to the PEr pool. 16:0/18:2-PE was equally distributed while 18:1/18:2-PE was 3-fold enriched in the PEr fraction. In all experiments, not only the PE but also the other phospholipids were separated and found to lack labeling of the fatty acid moieties, which indicates that transacylation did not influence the events described above. The results suggest that the type of fatty acid, both at the sn-1 and sn-2 positions on the PE, exerts a substantial influence on lipid distribution and localization in microsomal membranes.

DISCUSSION

In this study we have employed the amino reagent fluorescamine to investigate the structure and dynamics of PE in microsomal membranes. By using this probe we have demonstrated that compartmentalization can be obtained; this proved useful in the study of translocation and arrangement of lipids in microsomal membranes. The experimental findings suggest that PE in microsomes
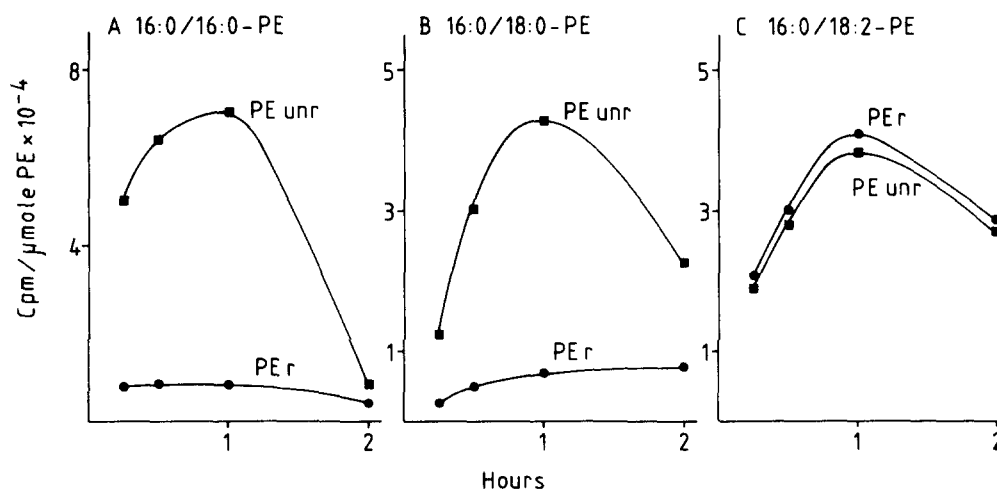

Fig. 5. Time course of uptake of injected PE into microsomal compartments; 16:0/[¹⁴C]16:0-PE (A), 16:0/[¹⁴C]18:0-PE (B), and 16:0/[¹⁴C]18:2-PE (C) in liposomal form were injected into the portal vein and their distributions were followed in microsomal PE after FLA treatment.

TABLE 5. Uptake of injected PE into microsomal compartments[a]

Injected PE	PEr	PEunr
	cpm/μmol × 10^{-3}	
16:0/16:0	9.7	68.5
16:0/18:0	7.2	43.8
16:0/18:1	9.0	20.2
16:0/18:2	41.1	40.8
18:1/18:2	60.3	21.5

[a] The distribution of the various labeled PE species after 1 hr of in vivo incorporation was followed in the two microsomal FLA compartments.

is distributed in a nonrandom fashion. The majority of the lipid was recovered in the pool which was easily accessible to FLA, while a minor part was found in the pool which in turn reacted slowly with the probe. Considerable quantitative and qualitative differences in fatty acid composition were found when the phospholipids in the two pools were investigated. The rate of incorporation of precursors into the compartments, under in vivo conditions, appeared to be specific both concerning the base and several of the fatty acids. Also, PE in liposomes injected into the portal vein was recovered in the microsomal membranes of the hepatocyte and was distributed unevenly among the pools as detected by reaction with FLA. These experiments point out some of the properties of the nonrandom distribution, not only involving the amount of the lipid in question, but also the amount and type of the fatty acids and the distribution of the newly synthesized molecules.

The existence of a compartment in which PE did not react with FLA was unexpected, since the probe easily penetrated microsomal membranes and rapidly appeared in the lumen of the vesicles. For this reason, it is unlikely that any major part of the PE would react with the probe only because of its localization on the outer surface (1).

An explanation for the incomplete fluorescamine reaction with microsomal PE is not available. Contamination with other intracellular structures of the microsomal fraction is obviously not the reason for the reaction pattern described. Cross linking experiments demonstrate that a considerable part of the PE in microsomes is preferentially associated with proteins (5). It is possible that PE in this association is not available for interaction with FLA. The fact that model membranes prepared from microsomal lipids in the absence of proteins possess only one single pool is in agreement with this hypothesis. It may also be expected that the numerous microsomal lipid-dependent enzymes require specific types of phospholipids for activation; however, this has not yet been proved (29). Finally, the individual steps of lipid biosynthesis are localized to the cytoplasmic surface of the membrane, implying an initially limited distribution of newly synthesized molecules (30, 31).

In the in vivo labeling experiments with glycerol and ethanolamine, and also with palmitic and stearic acid, a high specific labeling appeared in the PEr, as opposed to the PEunr pool, at early time points. After about 2-3 hr, equilibration of radioactivity between the two pools ensued. From these experiments one can conclude that a translocation of newly synthesized phospholipids is in process from the site of synthesis to a final location within the microsomal membrane. Since this translocation could not be detected under in vitro conditions, the probability exists that the intramembrane transport of PE is enzyme-mediated and that this process requires some factor that is not available under in vitro conditions.

The results of the studies of uptake and incorporation of in vivo injected labeled fatty acids supported the idea of compartmentalization. Another aspect of the findings was that the fate of the molecule in the hepatocytes could be studied. There is intensive discussion in the literature concerning the pathways of the fatty acids taken up in the course of phospholipid synthesis. They are utilized in de novo synthesis or in exchange reactions during the deacylation-reacylation process (28, 32, 33). Our results with the in vivo system emphasize the importance of the type of fatty acid, since the conclusion is that palmitic and stearic acids are mainly incorporated by de novo synthesis while deacylation-reacylation pathways play a major role for the incorporation of linoleic and arachidonic acid into the PE.

The ability for uptake of liposomes from the portal circulation during in vivo conditions is evidently a useful property of the hepatocyte. This approach opens the possibility of placing specifically labeled lipids in the microsomal membranes and of studying their distribution, configuration, and arrangement in the endoplasmic membranes. This procedure is experimentally effective, since the injected phospholipids are protected from hydrolytic breakdown before they become incorporated into the microsomal membrane. Applying radioautography to thin liver slices we could prove, as other investigators did (34), that hepatocytes in fact contain the labeled material after intraportal injection; that is, the label does not become localized exclusively in the Kupffer cells.

The experiments described in this report indicate that translocation of phospholipids is, at least in part, influenced by the constitutive fatty acids. The two FLA pools studied must exhibit a high degree of organization, since individual fatty acids in either pool are located mainly or exclusively in a certain position of the PE molecule. On the basis of this distribution, one can theoretically foresee that an entering lipid molecule prefers a compartment that fits its own fatty acid composition. In fact, PE in liposomes enters the hepatocyte and appears in that compartment of the microsomal membrane that contains lipids with a fatty acid composition similar to the injected one.
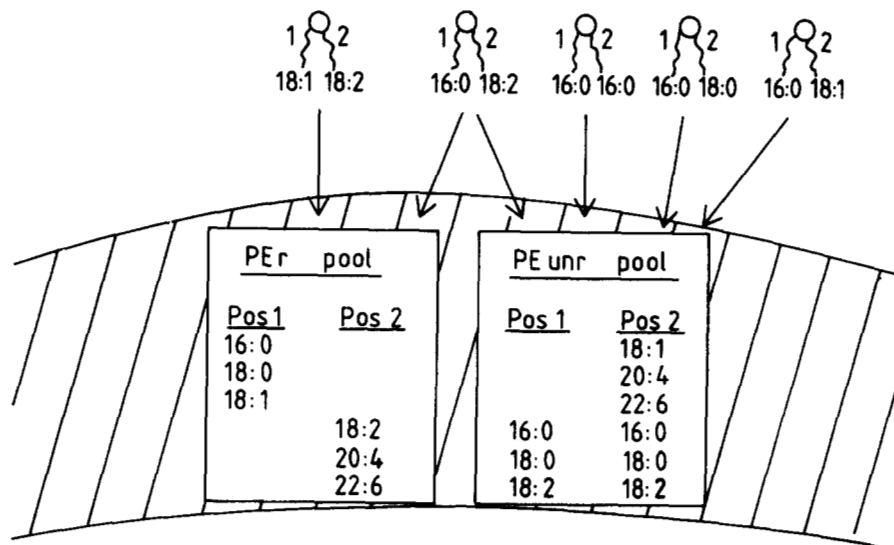
Fig. 6. Schematic picture of the influence of phospholipid fatty acid composition on the lipid targeting into microsomal compartments. The data in the two intramicrosomal PE pools represent the fatty acid composition in *sn*-positions 1 and 2, obtained from Table 3. The arrows at the outer surface of the membrane show the direction of incorporation of the specific phospholipid into one or the other pool under in vivo conditions. The results suggest that the fatty acid composition of the injected PE regulates the translocation of the lipid to the individual microsomal compartments.

According to this assumption, which is summarized in **Fig. 6,** the type and location of the fatty acid on the phospholipid has a signal function, directing the molecule to its appropriate final location. The results in the present study suggest that intramembrane transport may be mediated according to this principle. Such a pathway may be necessary for the direction of lipids from the site of synthesis to the place of their final location. Considering the cytoplasmic transport, it appears that the protein carriers themselves cannot mediate a full specificity of lipid transfer but have to be supported by additional mechanisms. Therefore, fatty acid content and distribution between the two positions of phospholipids may also provide signal information during the cytoplasmic transport among organelles. ▉

REFERENCES

1. Van Deenen, L. L. M. 1981. Topology and dynamics of phospholipids in membranes. *FEBS Lett.* **123:** 3–15.
2. Verkleij, A. J. 1984. Lipid intramembraneous particles. *Biochim. Biophys. Acta.* **779:** 43–63.
3. Valtersson, C., G. van Duijn, A. J. Verkleij, T. Chojnacki, B. De Kruijff, and G. Dallner. 1985. The influence of dolichol, dolichol esters and dolichyl phosphate on phospholipid polymorphism and fluidity in model membranes. *J. Biol. Chem.* **260:** 2742–2751.
4. Op den Kamp, J. A. F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* **48:** 47–71.
5. Valtersson, C., and G. Dallner. 1982. Compartmentalization of phosphatidylethanolamine in microsomal membranes from rat liver. *J. Lipid Res.* **23:** 868–876.
6. Rawyler, A., B. Roelofsen, and J. A. F. Op den Kamp. 1984. The use of fluorescamine as a permeant probe to localize phosphatidylethanolamine in intact Friend erythroleukaemic cells. *Biochim. Biophys. Acta.* **769:** 330–336.
7. Krebs, I. I. R., H. Hauser, and E. Carafoli. 1979. Asymmetric distribution of phospholipids in the inner membrane of beef heart mitochondria. *J. Biol. Chem.* **254:** 5308–5316.
8. Udenfriend, S., S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, protein, and primary amines in the picomole range. *Science.* **178:** 871–872.
9. Boss, W. F., C. J. Kelly, and F. R. Landsberg. 1975. A novel synthesis of spin label derivatives of phosphatidyl choline. *Anal. Biochem.* **64:** 284–291.
10. Smith, G. A., C. Montecucco, and J. P. Bennett. 1978. Isotopic labeling of phosphatidylcholine in the choline moiety. *Lipids.* **13:** 92–94.
11. Geurts Van Kessel, W. S. M., W. M. A. Hax, R. A. Demel, and J. DeGier. 1977. High performance liquid chromatographic separation and direct ultraviolet detection of phospholipids. *Biochim. Biophys. Acta.* **486:** 524–530.
12. Dallner, G. 1974. Isolation of rough and smooth microsomes—general. *Methods Enzymol.* **31A:** 191–201.
13. Ehrenreich, J. H., J. J. M. Bergeron, P. Siekevitz, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. *J. Cell Biol.* **59:** 45–72.
14. Coleman, R., R. H. Michell, J. B. Finean, and J. N. Hawthorne. 1967. A purified plasma membrane fraction isolated from rat liver under isotonic conditions. *Biochim. Biophys. Acta.* **135:** 573–579.

15. Leighton, F., B. Poole, H. Beufay, P. Baudhuin, J. W. Coffey, S. Fowles, and C. de Duve. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from livers of rats injected with Triton WR-1339. *J. Cell Biol.* **37:** 482–513.

16. Autuori, F., H. Svensson, and G. Dallner. 1975. Biogenesis of microsomal membrane glycoproteins in rat liver. I. Presence of glycoproteins in microsomes and cytosol. *J. Cell Biol.* **67:** 687–699.

17. Eriksson, L. C. 1973. Studies on the biogenesis of endoplasmic reticulum in the liver cell. *Acta Pathol. Microbiol. Scand. Suppl.* **239:** 1–72.

18. Marinetti, G. V., and K. Cattieu. 1982. Tightly (covalently) bound fatty acids in cell membrane proteins. *Biochim. Biophys. Acta.* **685:** 109–116.

19. Markides, K., L. Blomberg, S. Hoffmann, J. Buijten, and T. Wännman. 1984. Cyanosilicones as stationary phases in gas chromatography. *J. Chromatogr.* **302:** 319–340.

20. Deems, R. A., and E. A. Dennis. 1975. Characterization and physical properties of the major form of phospholipase A_2 from cobra venom (*Naja naja naja*) that has a molecular weight of 11,000. *J. Biol. Chem.* **250:** 9008–9012.

21. Kreibich, G., P. Debey, and D. D. Sabatini. 1973. Selective release of content from microsomal vesicles without membrane disassembly. *J. Cell Biol.* **58:** 436–462.

22. Nilsson, O. S., and G. Dallner. 1977. Enzyme and phospholipid asymmetry in liver microsomal membranes. *J. Cell Biol.* **72:** 568–583.

23. DePierre, J., and G. Dallner. 1976. Isolation, subfractionation and characterization of the endoplasmic reticulum. *In* Biochemical Analyses of Membranes. A. H. Maddy, editor. John Wiley and Sons, Inc., New York. 79–131.

24. Adelman, M. R., G. Blobel, and D. D. Sabatini. 1974. Non-destructive separation of rat liver rough microsomes into ribosomal and membraneous components. *Methods Enzymol.* **31A:** 201–215.

25. Autuori, F., H. Svensson, and G. Dallner, 1975. Biogenesis of microsomal membrane glycoproteins in rat liver. II. Purification of soluble glycoproteins and their incorporation into microsomal membranes. *J. Cell Biol.* **67:** 700–714.

26. Bergman, A., and G. Dallner. 1976. Distribution of protein-bound sugar residues in microsomal subfractions and Golgi membranes. *Biochim. Biophys. Acta.* **433:** 496–508.

27. Åkesson, B. 1970. Initial esterification and conversion of intraportally injected [1-^{14}C]linoleic acid in rat liver. *Biochim. Biophys. Acta.* **218:** 57–70.

28. Infante, J. P. 1984. Biosynthesis of acyl-specific glycerophospholipids in mammalian tissues. *FEBS Lett.* **170:** 1–14.

29. Winqvist, L., and G. Dallner. 1976. Localization of enzymes in specialized regions of the microsomal membrane. *Biochim. Biophys. Acta.* **436:** 399–412.

30. Vance, D. E., P. C. Choy, S. B. Farren, P. H. Lim, and W. J. Schneider. 1977. Asymmetry of phospholipid biosynthesis. *Nature.* **270:** 268–269.

31. Coleman, R., and R. M. Bell. 1978. Evidence that biosynthesis of phosphatidylethanolamine, phosphatidylcholine and triacylglycerol occurs on the cytoplasmic side of microsomal vesicles. *J. Cell Biol.* **76:** 245–253.

32. Van Golde, L. M. G., G. L. Scherphof, and L. L. M. Van Deenen. 1969. Biosynthetic pathways in the formation of individual molecular species of rat liver phospholipids. *Biochim. Biophys. Acta.* **176:** 635–637.

33. Sprecher, H., and P. M. Duffy. 1974. The biosynthesis and turnover of different molecular species of rat testicular choline phosphoglycerides and triacylglycerols following intratesticular injection with [1(3)-^{14}C]glycerol. *Biochim. Biophys. Acta.* **380:** 21–30.

34. Roerdink, F., J. Regts, B. Van Leeuwen, and G. Scherphof. 1984. Intrahepatic uptake and processing of intravenously injected unilamellar phospholipid vesicles in rats. *Biochim. Biophys. Acta.* **770:** 195–202.