

Compartmentalization of phosphatidylethanolamine in microsomal membranes from rat liver

Conny Valtersson and Gustav Dallner

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, and Department of Pathology at Huddinge Hospital, Karolinska Institutet, Stockholm, Sweden

Abstract Microsomal membranes from rat liver were treated with the cross-linking reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB). Experimental work showed that at a probe concentration of 0.75 mM all free phosphatidylethanolamine (PE) and phosphatidylserine (PS) were found as dinitrophenyl derivatives: 29% of PE was in monomeric form, 9% dimeric, 2% interacted with PS, and 63% cross-linked to protein. PS showed a greater percent in monomeric and dimeric form and only 31% was cross-linked to protein. The cross-linking pattern of PE was clearly different from that pattern which is present in the inner mitochondrial and erythrocyte membranes. In vivo labeling of PE with [³H]glycerol and [³H]ethanolamine followed by phospholipase A₂ treatment of isolated microsomes established a heterogeneous labeling pattern during the first 2 hours. During this period, the specific activity of the phospholipase A₂-sensitive compartment was considerably higher. The differential distribution of radioactivity after in vivo labeling in the part of the PE which reacted with increasing concentrations of DFDNB also indicated compartmentalization. After in vivo labeling with the precursors, the time course of the specific radioactivity demonstrated an initial high labeling, almost exclusively in the monomeric form, followed by a later appearance of the label in the protein-bound PE. The experiments indicate that the biosynthesis of PE takes place in a compartment that is more accessible to surface probes and that the labeled molecules are transferred in a time-dependent process to a second compartment where the lipid is not available for phospholipase A₂ action but is available for cross-linking to protein.—**Valtersson, C., and G. Dallner.** Compartmentalization of phosphatidylethanolamine in microsomal membranes from rat liver. *J. Lipid Res.* 1982. 23: 868–876.

Supplementary key words cross-linking • nearest neighbor analysis • phospholipase effect • in vivo labeling

The membranes of the liver endoplasmic reticulum (ER) have a central role in the cellular metabolism since they contain a large number of oxidative, reductive, and hydrolytic enzymes (1). They also produce macromolecules used for synthesis and renewal of intracellular membranes and for secretion (2). The large majority of phospholipid synthesis is localized in the ER membranes and the synthesizing enzymes are located on the cytoplasmic surface (3, 4). The distribution, molecular arrangement, and mobility of the microsomal phospholip-

ids has been studied in detail during recent years and the results are often contradictory (5). This stems from the fact that phospholipids are synthesized for different purposes and subsequently transported in three different directions: for microsomal membranes (6), for different intracellular organelles (7, 8), and for secretory lipoproteins synthesized in the lumen of the endoplasmic channels (9). Consequently, this complexity creates difficulties when one is searching for uniformity of structural and functional properties.

Various cross-linking reagents have previously been applied with success for analysis of various membranes and valuable information has been obtained concerning the structural organization of intramembranous phospholipids (10–15). A test of the various cross-linking reagents for nearest neighbor analysis has indicated that one of the few probes that can be used for microsomes is DFDNB. This study describes the interaction of DFDNB with microsomal aminolipids. This interaction gives a specific pattern of cross-linking not apparent in other types of membranes.

MATERIALS AND METHODS

Animals

Adult male rats with a body weight of 180–200 g were used after overnight starvation. [²⁻³H]Glycerol (500 mCi/mmol) or [³H]ethanolamine (16.5 Ci/mmol) from Radiochemical Centre, Amersham, was injected either intraperitoneally or intraperitoneally. In the case of injection into the portal vein, NaCl was added to the radioactive solution to a final concentration of 0.9% and 0.2 ml of the solution was injected under Nembutal (6 mg/100 g body weight) anaesthesia.

Abbreviations: PL, phospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; FDNP, fluoro-2,4-dinitrophenyl; DNP, 2,4-dinitrophenyl; PLase A₂, phospholipase A₂.

Fractionation

Total microsomes from rat liver were prepared as described earlier (16). Inner mitochondrial membranes were isolated by the procedure of Sottocasa et al. (17). Human erythrocyte ghosts were prepared as described by Marinetti and Love (10).

Treatment with phospholipase A₂

Phospholipase A₂ was isolated from *Naja naja* venom (Sigma) as described by Deems and Dennis (18). The solubilized acid precipitate was chromatographed first on CM-Sepharose and then on Sephadex G-100. The specific activity of the PLase A₂, measured as described earlier (19), was 1180 IU/mg of protein.

Microsomes were suspended in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.25 M sucrose to give a protein concentration of 5 mg/ml. Purified PLase A₂ was added to the incubation medium (2 IU/ml) together with 1 mM CaCl₂ and 40 mg bovine serum albumin/ml, and incubated at 0°C. The reaction was stopped by addition of 20 mM EDTA and the mixture was diluted with 0.25 M sucrose before ultracentrifugation. The pellet was extracted and analyzed as described below.

DFDNB treatment

In the cross-linking experiments, the incubation medium contained 40 mM NaCl, 120 mM NaHCO₃, pH 8.5, 8 mM EDTA, 0.25 M sucrose, 0.75 mM DFDNB (Pierce), (unless otherwise indicated), and an aliquot of membrane fraction. In routine experiments, 10 mg of microsomal protein was present in a final volume of 4 ml. Incubation was performed at 20°C for 16 hr and these results are given in the tables and figures. The experiments were also performed with 2-hr incubation periods and the results obtained were practically identical with those with the longer incubation time. At the end of the incubation, the medium was diluted with 5 ml of cold 0.25 M sucrose and centrifuged at 105,000 *g* for 45 min.

Lipid extraction and analysis of the protein residue

The membrane pellets were resuspended in 0.25 M sucrose, precipitated with cold 6% trichloroacetic acid, and were washed once with the acid and once with cold water. Extraction was with chloroform-methanol 2:1 at 30°C for 1 hr in the presence of 0.05% butylated hydroxytoluene and under N₂ atmosphere. The protein residue remaining after lipid extraction was washed twice with chloroform-methanol 2:1 and digested with perchloric acid; this was used for phosphate measurements. The re-washed pellet was dissolved in 1 ml of 2% sodium dodecylsulfate at 45°C overnight. After addition of 10 ml of Aqualuma Plus scintillator (Lumac,

Holland) the solution was thoroughly mixed with a Tur-rax blender. One ml from this mixture was diluted with 10 ml of Aqualuma Plus and the radioactivity in the protein was measured in a scintillation counter.

The protein was determined in the membrane fractions before incubation with the probe or before PLase A₂ treatment by the biuret procedure (20).

Lipid separation and analysis

The chloroform-methanol lipid extracts were partitioned with 5 ml of cold 0.1 M HCl and aliquots of the chloroform extract were dried under N₂. The lipids were dissolved in 20 μl of chloroform and applied on silica gel 60 plates (Merck) for two-dimensional thin-layer chromatography. Individual phospholipids, extracted from control microsomes, were separated by developing the plates in chloroform-methanol-acetic acid-water 106:50:12:6 (v/v) in the first direction and with chloroform-acetone-methanol-acetic acid-water 50:20:10:10:5 (v/v) in the second direction.

When lipid extracts from DFDNB-treated membranes were subjected to chromatography, the silica plates were developed with chloroform-methanol-water 130:45:8 (v/v) in the first direction and with chloroform-acetone-methanol-acetic acid-water 50:20:10:10:5 (v/v) in the other direction.

The various dinitrophenyl-lipid derivatives were identified by several methods. Liposomes of synthetic lecithin containing PE or/and PS were prepared and treated with DFDNB and the derivatives were separated as above. PE was labeled by in vivo injection of [³H]ethanolamine which only gave labeling in the PE derivatives. The individual dinitrophenyl derivatives were also extracted and the hydrolyzed ethanolamine and serine derivatives were identified by chromatography according to Marinetti and Love (10).

A plate with the separated dinitrophenyl-lipid derivatives is shown in Fig. 1.

Lipid-phosphorus

The chromatographed lipid spots were visualized by iodine vapor, scraped off, and hydrolyzed overnight in a sand bath in the presence of 1 ml of perchloric acid. Aliquots were evaporated by heating. After addition of 50 μl of 5N H₂SO₄ and 0.2 ml of H₂O, the tubes were placed in a boiling water bath for 10 min. Water (0.5 ml), 50 μl of 2.5% ammonium molybdate, and 50 μl of 10% ascorbic acid were added. After heating the sample at 45°C for 20 min, the absorbance was measured at 820 nm. The same procedure was employed to measure the amount of phosphate in the protein hydrolysate.

When radioactivity was determined in the individual spots, the iodine vapor-colored lipid derivatives were scraped into scintillation vials, 1 ml of methanol was

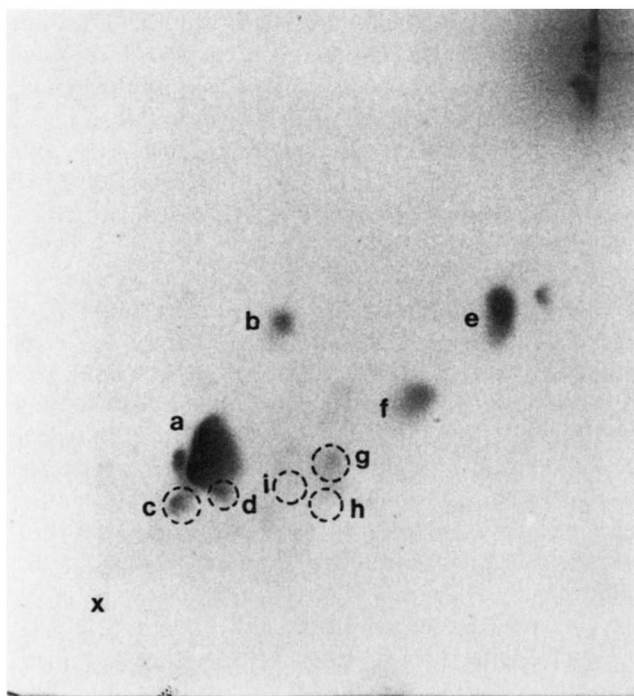


Fig. 1. Separation of dinitrophenyl derivatives of microsomal aminophospholipids after two-dimensional thin-layer chromatography. a), Phosphatidylcholine; b), phosphatidylethanolamine; c), phosphatidylinositol and sphingomyelin; d), phosphatidylserine; e), FDNP-PE; f), PE-DNP-PE; g), FDNP-PS; h), PS-DNP-PE; i), PS-DNP-PS; x), origin.

added and the mixture was heated at 50°C for 1 hr. Ten ml of Aqualuma Plus was added and the radioactivity was measured in a scintillation counter.

RESULTS

Incubation with DFDNB

The microsomal membranes of rat liver used in this investigation were washed thoroughly in order to remove

the large amount of absorbed basic cytoplasmic proteins (30%) and luminal secretory protein (20%) (21). The membranes are rich in phospholipids and have a phospholipid/protein ratio around 0.40. The dominating lipid is PC which makes up half of the total. PE is the aminolipid in greatest abundance, while PS is only 5% of total. Previous investigations established that the two aminolipids are constitutive components of the microsomal membranes and not the results of the limited cross-contamination from other organelles present in this fraction (21–23). The PL/protein ratio and the phospholipid composition in rough and smooth microsomes are identical and for this reason these subfractions were not separated in the following experiments.

Incubation of microsomes with DFDNB at 20°C in alkaline buffer gives typical titration curves similar to those described for erythrocytes (10). At a protein concentration of 10 mg/ml, it was necessary to have a concentration of 0.75 mM DFDNB in the incubation medium to obtain a complete interaction of all PE (**Fig. 2**). This concentration also gave the maximal interaction between PE and protein but not between PE and PE. Maximal cross-linking of PE to PE could be obtained at 0.25 mM DFDNB. At this level, 40% of the PE was in dimeric form. As expected, increased concentrations of the reagent gave a continuous increase in the amount of monomers and a decrease in the amount of dimers, the latter being only 9% at 0.75 mM DFDNB concentration. Cross-linking of PE to PS occurs only in very limiting amount in microsomes and does not exceed 2% of the total.

The picture was similar with PS (**Fig. 3**). Complete interaction of PS with the probe required 0.75 mM DFDNB during incubation and this concentration also gave maximal cross-linking of PS both to PS and to protein. As described in **Fig. 2**, PS interaction with PE took place to a small extent.

The type of interaction of PE and PS with the bi-

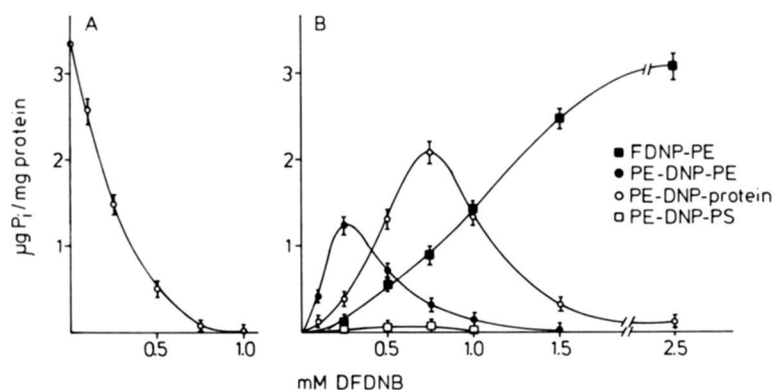


Fig. 2. Interaction of microsomal PE with different concentrations of DFDNB. a), Unreacted PE; b), reacted PE. The values are the result of seven experiments; the vertical bars represent SEM.

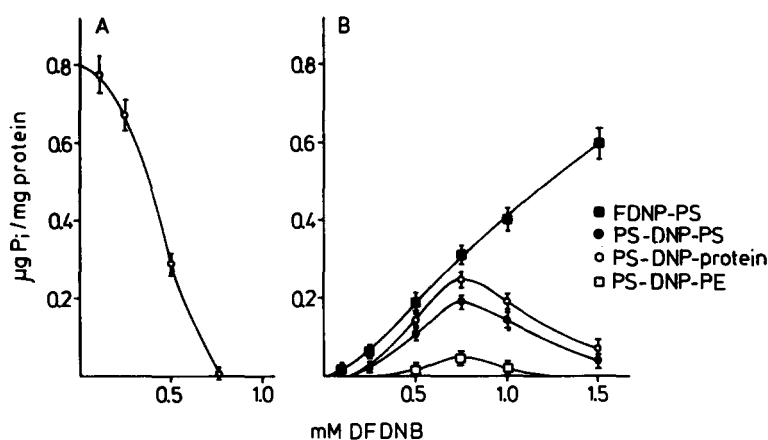


Fig. 3. Interaction of microsomal PS with different concentrations of DFDNB. a), Unreacted PS; b), reacted PS. The values are the result of five experiments; the vertical bars represent SEM.

functional reagent when all the aminolipids are reacted is shown in Table 1. Under the conditions used, as much as two-thirds of the total PE was present in the PE-DNP-protein form and the majority of the remaining aminolipid (26%) was in monomeric form. Cross-linking of PS to protein was significantly lower and made up only 30% of the total. The remaining PS was present in monomeric and dimeric forms.

PE occurs in sizeable quantities in all intracellular membranes and is clearly of great biological interest. For this reason, the cross-linking pattern of microsomes was compared with that of the inner mitochondrial membranes and erythrocyte ghosts (Table 2). The titration curve which is described in Fig. 2 was also performed with these membranes. At DFDNB concentrations that gave maximal cross-linking of the PE to protein, only 28% of the mitochondrial and 4% of erythrocyte PE reacted with protein. The results with erythrocytes are in agreement with those found previously by Marinetti and Love (24). These membranes are also characterized by

a sizeable interaction of PE with PS. The experiments strongly suggest that the cross-linking pattern described reflects lipid arrangement in membranes and not the result of a random interaction governed by phospholipid amount or composition. PE is present in similar amounts in microsomal, inner mitochondrial, and erythrocyte membranes (28, 34, and 28% of total PL, respectively) and, in spite of this, its cross-linking pattern is very different.

Effect of phospholipase A₂

In order to study the possible compartmentalization of microsomal PE, isolated vesicles were subjected to phospholipase A₂ treatment. Under our conditions the treated vesicles kept their permeability barrier intact, as judged by the analysis of mannose-6-phosphatase and nucleoside diphosphatase, and by estimation of the intramicrosomal water space (25, 26). Since the permeability barrier was not destroyed, it was assumed that at least some aspect of membrane structure integrity was unchanged and therefore the hydrolytic products did not represent a random removal, but might have originated

TABLE 1. Cross-linking of aminophospholipids in microsomal membranes^a

Derivative	Distribution
	% of total PE
PE as FDNP-PE	26 ± 3
PE-DNP-PE	9 ± 1
PE-DNP-PS	2 ± 0.3
PE-DNP-protein	63 ± 5
PS as FDNP-PS	39 ± 2
PS-DNP-PS	27 ± 3
PS-DNP-PE	6 ± 1
PS-DNP-protein	31 ± 2

^a The incubation mixture contained 0.75 mM DFDNB which gave maximal interaction of PE with protein. At this concentration all the PE and PS reacted. The values present the means of seven experiments ±SEM.

TABLE 2. Cross-linking of aminophospholipids in microsomal, inner mitochondrial, and erythrocyte membranes^a

Derivative	Microsomes	Inner Mitochondrial Membranes		Erythrocytes
		% of total PE		
PE as FDNP-PE	26 ± 4	39 ± 3	25 ± 2	
PE-DNP-PE	9 ± 1	32 ± 4	48 ± 6	
PE-DNP-PS	2 ± 0.3	1 ± 0.1	23 ± 3	
PE-DNP-protein	63 ± 7	28 ± 3	4 ± 0.5	

^a The incubation mixture contained 0.75 mM DFDNB in the case of microsomes, 0.5 mM for mitochondria, and 0.1 mM DFDNB for erythrocytes. At these concentrations all the membrane PE reacted with the probe and a maximal cross-linking of protein to PE was apparent. The values present the means of three experiments ±SEM.

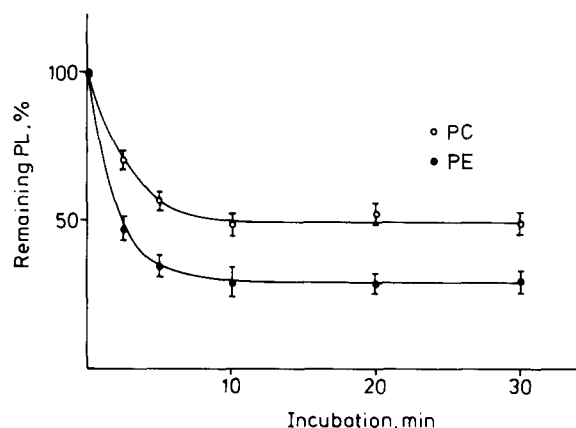


Fig. 4. PLase A₂ treatment of microsomes. Microsomes were incubated with purified PLase A₂ (0.5 IU/mg protein) in the presence of 1 mM CaCl₂ and 40 mg bovine serum albumin/ml. Incubations were conducted at 0°C for various times. The values give the phospholipid remaining in the membranes after centrifugation. The values represent the means of four experiments \pm SEM.

from a specific compartment available for phospholipase action. Under the conditions employed during the initial part of hydrolysis, about half of the PC and 70% of the PE were removed (Fig. 4). Prolonged incubation or increased amount of phospholipase A₂ did not solubilize that part of the phospholipids remaining attached to the membrane. Thus, a phospholipase-sensitive and an insensitive compartment can be arbitrarily established.

Labeling of microsomal PE in vivo was performed by using the precursors [³H]glycerol and [³H]ethanolamine. Although similar results with these two precursors were expected, some difference may be obtained because they are involved in several metabolic pathways in vivo, and ethanolamine can be exchanged by base-exchange reactions. During the initial 3-hr in vivo labeling period

with [³H]ethanolamine, the specific activity of PE in the PLase A₂-sensitive compartment was twice that in the PLase A₂-insensitive compartment (Fig. 5a). Equilibration occurred after 4 hr and the specific radioactivity was the same in the two compartments. The results obtained with [³H]glycerol were similar. Considerably higher specific activity was found in that part of PE which was removed by the hydrolytic action, and equilibration occurred after the initial 3 hr (Fig. 5b).

Treatment of microsomes with various DFDNB concentrations

A limited amount of DFDNB reacted with only a part of the free PE, providing a possible method to test compartmentalization. When the incubation medium contained 0.25 mM DFDNB, 42% of PE remained in non-reacted form and at increasing reagent concentrations (0.60 and 0.75 mM) the free PE decreased to 15% and 3%, respectively (Table 3). The specific radioactivity of PE decreased in parallel after 30 min in vivo labeling with [³H]ethanolamine (injected into the portal vein) and to a somewhat lesser extent after 120 min. As is shown in Fig. 2, at this concentration range the monomeric form increased and the amount of dimers decreased. The specific radioactivity of the monomer and the dimer at different DFDNB concentrations differed significantly. Increased concentration of DFDNB produced PE monomeric species with increased specific radioactivity while the dimeric species, under the same conditions, exhibited significantly decreased specific activities.

Labeling experiments with [³H]glycerol were also performed by using a different route for administration of radioactivity. Instead of being injected directly into the liver through the portal vein, [³H]glycerol was administered intraperitoneally, resulting in a lower and delayed

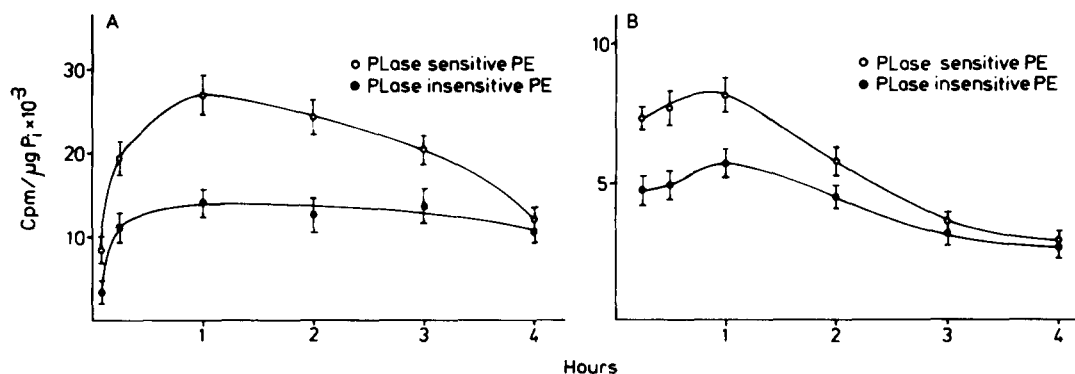


Fig. 5. PLase A₂ treatment of in vivo labeled microsomes. A), Rats were injected with 200 μ Ci of [³H]ethanolamine into the portal vein at various times before decapitation. The isolated microsomes were subjected to PLase A₂ treatment (0.5 IU/mg prot., 0°C, 15 min) and the specific radioactivity was determined in the PE isolated from the pellet after ultracentrifugation. Isolated control microsomes were also incubated in the absence of PLase A₂ and the amount of PE and radioactivity of the PE were determined. The values in the PLase A₂-sensitive part were calculated from the differences between the PE from nontreated microsomes and the PE remaining after enzymic hydrolysis. B), Rats were injected (portal vein) with 200 μ Ci of [³H]glycerol and the incubations and measurements were performed as in A). The values represent the means of four experiments \pm SEM.

TABLE 3. Cross-linking of in vivo [³H]ethanolamine-labeled microsomal PE in the presence of increasing concentrations of DFDNB^a

Treatment	PE			FDNP-PE			PE-DNP-PE		
	Amount	Sp Act		Amount	Sp Act		Amount	Sp Act	
		30 min	120 min		30 min	120 min		30 min	120 min
	$\mu\text{g Pi}$	$\text{cpm}/\mu\text{g Pi} \times 10^{-3}$		$\mu\text{g Pi}$	$\text{cpm}/\mu\text{g Pi} \times 10^{-3}$		$\mu\text{g Pi}$	$\text{cpm}/\mu\text{g Pi} \times 10^{-3}$	
None	31.6 ± 2.9	23.2 ± 2.6	37.4 ± 3.0	1.0 ± 0.1	6.9 ± 0.8	17.0 ± 1.4	11.9 ± 1.0	23.5 ± 2.6	32.2 ± 3.0
0.25 mM DFDNB	13.4 ± 1.3	16.3 ± 1.9	35.2 ± 3.2	1.0 ± 0.1	6.9 ± 0.8	17.0 ± 1.4	11.9 ± 1.0	23.5 ± 2.6	32.2 ± 3.0
0.60 mM DFDNB	4.7 ± 0.4	4.9 ± 0.6	22.3 ± 2.0	7.7 ± 0.9	14.1 ± 1.5	25.4 ± 1.9	4.0 ± 0.3	4.0 ± 3.2	30.5 ± 2.8
0.75 mM DFDNB	1.1 ± 0.1	1.3 ± 0.1	7.9 ± 0.1	9.1 ± 0.8	21.3 ± 1.8	38.2 ± 3.9	3.1 ± 0.3	3.1 ± 0.3	11.2 ± 0.2

^a Rats were injected (portal vein) with 200 μCi of [³H]ethanolamine 30 and 120 min before decapitation. Microsomes were isolated and treated (10 mg protein) with different concentrations of DFDNB. The specific radioactivity and phosphate (Pi) were measured in the spots after separation of the lipids by thin-layer chromatography. The values are the means \pm SEM (n = 5).

uptake of label. As shown in Table 4, even in this case, the specific activity of the remaining, nonreacted PE was decreased.

Time course of labeling after DFDNB treatment

At sufficiently high concentrations, DFDNB, under suitable conditions, interacts with proteins and lipids and gives two main PE compartments, FDNP-PE and PE-DNP-protein. In this way another type of compartmentalization was established in microsomal membranes and the time course of the labeling pattern was followed after pulse labeling with [³H]ethanolamine injected into the portal vein (Fig. 6). In these experiments, microsomes were isolated after various timepoints of in vivo labeling and treated with the probe to obtain the two major PE compartments: FDNP-PE and PE-DNP-protein. In the monomeric fraction there was a high initial labeling and the specific radioactivity exceeded several times that found in the PE cross-linked to protein. The radioactivity in the monomeric form was not decreased in the first 4-hr period probably because of the slow depletion of the pool or because of a continuous base exchange reaction.

TABLE 4. Cross-linking of in vivo [³H]glycerol-labeled microsomal PE in the presence of increasing concentrations of DFDNB^a

Treatment	PE		
	Amount	Sp Act	
		30 min	120 min
	$\mu\text{g Pi}$	$\text{cpm}/\mu\text{g Pi}$	
None	30.7 ± 2.6	2230 ± 170	958 ± 72
0.25 mM DFDNB	12.6 ± 0.9	1780 ± 162	702 ± 49
0.60 mM DFDNB	3.6 ± 0.3	1310 ± 141	569 ± 58

^a Rats were injected intraperitoneally with 400 μCi of [³H]glycerol 30 and 120 min before decapitation. Experiments with microsomes were performed as described in Table 3. The values are means \pm SEM (n = 5).

The experiments with [³H]glycerol in vivo also demonstrated a differential labeling, with time, of the two main compartments (Fig. 7). In the initial period there was a high labeling of the monomeric form while that of the PE-DNP-protein fraction was almost unlabeled. In this latter form, the specific activity increased in the first 1–2 hr, while the specific activity of the monomeric form rapidly decreased to a value significantly below that of the PE cross-linked to protein. These experiments verified the metabolic heterogeneity of two PE species in microsomal membranes.

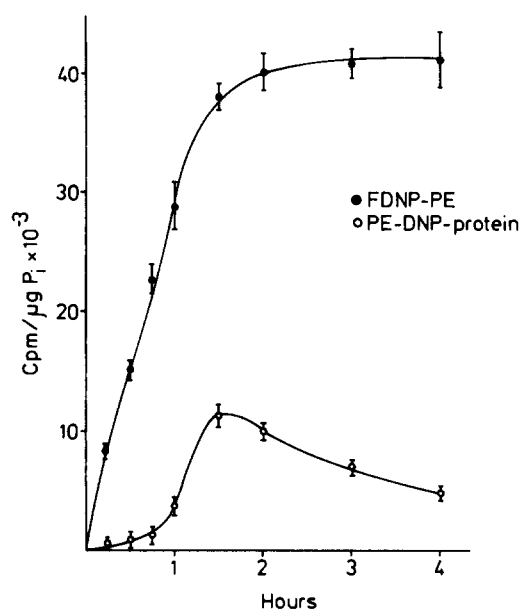


Fig. 6. Distribution of [³H]ethanolamine in microsomal PE derivatives after DFDNB treatment. Rats were injected with 200 μCi of [³H]ethanolamine into the portal vein and decapitated at various times. The isolated microsomes were treated with 0.75 mM DFDNB (10 mg protein/4 ml, 20°C, 16 hr) and the lipids were extracted and separated by two-dimensional thin-layer chromatography. The radioactivity and the phosphate in the monomeric derivative of PE were measured. The radioactivity and phosphate were also determined in the extracted protein pellet. The values are the means \pm SEM (n = 5).

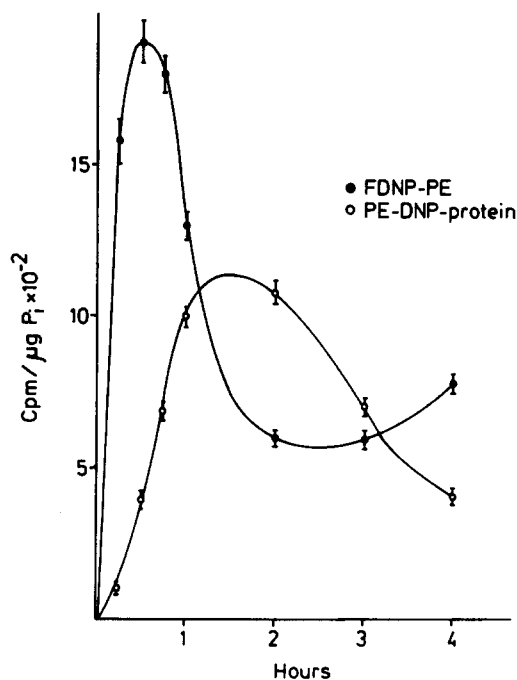


Fig. 7. Distribution of [³H]glycerol in microsomal PE derivatives after DFDNB treatment. Rats were injected into the portal vein with 200 μCi of [³H]glycerol and the experiment and measurements were performed as described in Fig. 6. The values are the means ± SEM (n = 5).

DISCUSSION

Although the lipid structure of liver microsomal membranes has been extensively studied in recent years, the details of intramembrane distribution, molecular organization, and mobility are still unclear. Studies using the cross-linking agent DFDNB provided us with a valuable tool to answer questions about lipid organization.

DFDNB penetrates microsomal membranes and a characteristic cross-linking pattern was found after 16 hr of incubation. Essentially the same results were obtained after 2 hr and it does not appear that the results obtained were influenced by membrane deterioration as a consequence of the long incubation time. The fact that the first fluorine of this probe reacts with amino groups faster than the second fluorine (24) does not appear to influence the cross-linking pattern. The arrangement of aminolipids within the membrane seems to be the primary explanation for the pattern obtained. The dinitrophenyl derivatives of the whole phospholipids could be separated by thin-layer chromatography with a relatively easy and reproducible procedure. The values obtained in this way were the same as those obtained by isolation of dinitrophenyl derivatives of the bases after hydrolysis.

Using appropriate probe concentration, a large amount

of dimers of PE was obtained indicating clustering of these phospholipids in the bilayer. At the concentration that allowed all the PE to react, a large part of the lipids occurred in a protein-bound form. It was shown previously that certain microsomal proteins immobilize a part of the membrane phospholipids (27) and it is possible that the PE cross-linked to protein in our experimental conditions represents a part of the immobilized membrane lipids. It would be of importance to establish which membrane proteins are the nearest neighbors to PE, but our efforts to identify these proteins were unsuccessful because of the extensive cross-linking between proteins. Interestingly, nearest neighbor analysis has shown that PE can be cross-linked to PS to a limited extent. PS occurs only in small amounts in microsomal membranes and appears to be predominantly clustered and associated with proteins only to a limited extent. The pattern described for DFDNB interaction with aminolipids in microsomal membranes suggests a unique organization quite different from that found in inner mitochondrial membranes and erythrocytes. Also, previous investigations on platelets demonstrated a cross-linking pattern which was different from that observed in microsomes (15). Concerning microsomes, one could raise the possibility that preparation of these vesicles involves fragmentation of the endoplasmic reticulum and leads to reorganization of the original structure, and also, the cross-linking pattern with DFDNB. Detailed studies on permeability, surface charge, interaction with fluorescence probes, and enzymic pattern have demonstrated previously that the isolated microsomal vesicles possess membrane properties that exclude any major damage caused by the preparation procedure (1). For this reason, it appears improbable that the results obtained by DFDNB are artifacts, and therefore they are different from the results obtained with erythrocytes. The fact that the PS-DNP-PS and PS-DNP-protein derivatives that are formed in microsomal membranes are similar to those in erythrocytes, while PE derivatives of the two membranes are different, also speaks against such artifacts. Albumin in the lumen of the vesicles may contain some PE which could alter the results in experiments of the type performed in this investigation. However, we made a number of studies with vesicles permeable to macromolecules and consequently free of secretory proteins. The results with DFDNB were identical to those described above.

Analysis with cross-linking reagents may be useful in detecting subtle changes in membrane lipid organization. Recently, we have performed analyses on microsomal membranes induced by phenobarbital and methylcholanthrene. It was found that the lipid composition, quantitative and qualitative, was unchanged in microsomes,

but the amount of PE that could be cross-linked to proteins was decreased (28).

The concept of membrane fluidity is generally accepted, but the extent and time course of lipid mobility is not established. Obviously, the approach employed is critical. In our experiments we tried to establish compartmentalization in relation to membrane structure and to follow the labeling patterns after in vivo injection of radioactive precursors. Membrane PE in the PLase A₂-sensitive and nonsensitive compartments, in both the DFDNB-reacted and nonreacted form, displayed different specific activities in the first 2 hr. Later, an equilibration was reached. The simplest interpretation of these results is that sizeable movements of lipids occur within and also between compartments taking as long as several hours under in vivo conditions. Other investigations have also indicated compartmentalization of PI in microsomes (29), of PE in erythrocytes (30) and inner mitochondrial membrane (31), and of PC in erythrocytes (32, 33).

The compartments in the microsomal membranes which were arbitrarily established by incubation of microsomes with DFDNB after in vivo labeling were also useful in following the appearance and turnover characteristics of PE. The high label in the monomeric form, in contrast to the protein-bound form, cannot be explained by simple dilution of the newly synthesized lipids in the latter fraction. There are two possible explanations. The first is that the lipid is synthesized at only one location and is translocated slowly to the other compartment; the second explanation is that there are two separate locations for PE synthesis with two different rates of synthesis. The gradual increase of specific activity in the protein-bound form and the decrease in the monomeric fraction support the first hypothesis. Since phosphatidylserine decarboxylation occurs only in mitochondria (34) and base exchange resulting in PE is a relatively limited process (35), the major pathway for PE is de novo synthesis through diacylglycerol-ethanolamine-phosphotransferase. This enzyme, together with the initial reactions resulting in acylation of glycerol 3-phosphate, is present on the outer surface of microsomes (3, 4) and this newly synthesized pool is probably easily available to PLase A₂, and also for DFDNB to produce monomers. The newly synthesized PE from here may be transferred in a time-dependent process to the second compartment where it is no longer available to PLase A₂ but can be cross-linked to protein. The process of translocation between two compartments requires 1–2 hr. Establishment of the exact time course of lipid relocation in microsomal membrane will require further labeling experiments with living cells as well as cross-linking or other types of immobilization in vivo before isolation. ■

This work was supported by grants from the Swedish Medical Research Council.

Manuscript received 20 November 1981 and in revised form 27 April 1982.

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