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PHOSPHATIDYLCHOLINE MOBILITY IN LIVER MICROSOMAL MEMBRANES

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Summary

Purified phosphatidylcholine exchange protein from bovine liver was used to exchange rat liver microsomal phosphatidylcholine for egg phosphatidylcholine. It was found that at 25 and 37°C rat liver microsomal phosphatidylcholine was completely and rapidly available for replacement by egg phosphatidylcholine. In contrast, phosphatidylcholine in vesicles prepared from total microsomal lipids could only be exchanged for about 60%. At 8 and 0°C complex exchange kinetics were observed for phosphatidylcholine in rat liver microsomes. The exchange process had neither effect on the permeability of the microsomal membrane to mannose 6-phosphate, nor on the permeability of the phosphatidylcholine vesicles to neodymium (III) cations.

Purified phospholipase A_2 from *Naja naja* could hydrolyze some 55—60% of microsomal phosphatidylcholine at 0°C, but 70—80% at 37°C. Microsomal phosphatidylcholine, remaining after phospholipase treatment at 37°C, could be exchanged for egg phosphatidylcholine at 37°C, but at a slower rate than with intact microsomes. Microsomal phosphatidylcholine remaining after phospholipase treatment at 0 and 37°C had a lower content of arachidonic acid than the original phosphatidylcholine.

These results are discussed with respect to the localization and transmembrane movement of phosphatidylcholine in liver microsomes.

Introduction

A current model for biological membranes is a (phospho)lipid bilayer in which proteins are embedded [1]. Artificial bilayers of pure (phospho)lipids have been studied extensively as model systems for biological membranes. In these artificial systems the phospholipid molecules can perform only restricted movements. In the liquid-crystalline state diffusion in the plane of the bilayer is

a fast process with a diffusion constant in the order of 10^{-7}—10^{-8} $cm^2 \cdot s^{-1}$ [2,3]. However, movement from one side of the bilayer to the other (flip-flop) appears to be extremely slow with half times in the order of several days [4—6].

Evidence for an asymmetric transverse distribution of phospholipids has been presented for several biological membranes (see ref. 7 for a recent review). Nilsson and Dallner [8,9] presented an asymmetric distribution of the phospholipids in rat liver microsomal membranes, based on phospholipase A_2 degradation studies.

The purpose of the present study is to obtain information on the dynamics of phospholipid molecules in microsomal membranes. Phosphatidylcholine exchange protein from bovine liver was utilized to determine the pool size of exchangeable microsomal phosphatidylcholine. The phospholipase A_2 degradation studies by Nilsson and Dallner were repeated and extended.

Materials and Methods

Animals. Adult male Wistar rats were injected intraperitoneally with 0.5—1.0 mCi of carrier-free, isotonic sodium[^{32}P]phosphate (Philips-Duphar, The Netherlands) 16 h before killing. The rats were starved after injection.

Preparation and analysis of microsomes. Livers were homogenized in nine volumes of 0.25 M sucrose, 10 mM Tris · HCl (pH 7.2) and centrifuged for 15 min at 600 × *g*. The supernatant was centrifuged for 20 min at 16 000 × *g*. The resulting supernatant was carefully removed and centrifuged for 1 h at 100 000 × *g*. The pellet was resuspended in 0.15 M Tris · HCl (pH 8.0) and centrifuged for 1 h at 100 000 × *g* to remove adsorbed proteins. The pellet was resuspended in a buffer containing 0.25 M sucrose or 0.15 M NaCl.

Protein content was determined according to Lowry et al. [10]. Lipids were extracted according to Bligh and Dyer [11]. Phospholipids were separated by two-dimensional thin-layer chromatography according to Broekhuyse [12]. Spots were scraped and extracted with methanol. Phosphorus was determined according to Chen et al. [13] after destruction of the sample as described by Ames and Dubin [14]. Phospholipid fatty acyl residues were analyzed after esterification with methanol according to Morrison and Smith [15]. Fatty acid methyl esters were dissolved in 2,2,4-trimethylpentane and separated in a Packard Becker gas chromatograph (model 419) using a column of 12% polyethyleneglycol adipate on Gaschrom Q (Applied Science). The column temperature was 195°C.

Lipids and preparation of vesicles. Phosphatidylcholine was isolated from egg yolks according to the method of Papahadjopoulos and Miller [16]. [1α, 2α(n)-$^3H_2$]Cholesteryl oleate was prepared as described previously [17]. Lipids were mixed at the desired ratio in chloroform. The solvent was evaporated in vacuo. The dry lipid was suspended in buffer solution by mechanical agitation with glass beads. The suspension (2—5 ml) was sonicated under nitrogen at 0—10°C for 30 min using a Branson Sonifier (energy output, 50 W). After sonication, the solution was centrifuged at 150 000 × *g* for 2 h. The upper part of the supernatant was pipetted off as described by Barenholz et al. [18] and used for the experiments.

Phosphatidylcholine exchange assay. Phosphatidylcholine exchange protein

was purified from beef liver according to Kamp et al. [19]. It was stored in 50% glycerol at −20°C. Before use it was dialyzed against the incubation buffer. Transfer of [^{32}P]phosphatidylcholine from microsomes to sonicated egg phosphatidylcholine vesicles was measured as described by Kamp et al. [19]. ^{32}P-labeled microsomes were incubated with sonicated egg phosphatidylcholine vesicles labeled with [^{3}H]cholesteryl oleate (0.03 mol %) in a total volume of 1 ml. At the end of the incubation the mixture was chilled in ice and the pH of the medium adjusted to 5.0 by addition of 2 ml 0.2 M sodium acetate/acetic acid (pH 5.0). Microsomes were sedimented at 16 000 rev./min for 10 min in the SS34 rotor of the Sorvall centrifuge in Sorvall tubes (No. 250) with adapters. The supernatant containing the sonicated vesicles was collected and the lipids extracted according to Bligh and Dyer [11]. In general the recovery of ^{3}H label was 80—90%. The extract was concentrated to dryness under a stream of nitrogen and dissolved in 16 ml of toluene (0.5% PPO, 0.03% POPOP). Radioactivity was measured with a Packard Tricarb liquid scintillation spectrometer. The relative efficiency was determined by the channel ratio method and the external standard method. The ^{32}P/^{3}H ratio was determined. Since the exchange protein transfers specifically phosphatidylcholine, this ratio was used to calculate the transfer of [^{32}P]phosphatidylcholine from the microsomes to the vesicles. In some cases the vesicle extract was analyzed by two-dimensional thin-layer chromatography [12] to determine the ^{32}P radioactivity in the individual phospholipid spots. Incubation of [^{3}H]cholesteryl oleate incorporated in egg phosphatidylcholine vesicles with microsomes for 60 min at 37°C did not result in more than 10% hydrolysis of the cholesteryl oleate. Total ^{3}H recovery remained constant also for long term incubations.

Phosphatidylcholine exchange between two vesicle populations. Phosphatidylcholine exchange between two populations of sonicated vesicles was assayed according to van den Besselaar et al. [17]. Sonicated vesicles of total lipids from ^{32}P-labeled rat liver microsomes were prepared in 12.5 mM potassium phosphate/1 mm EDTA/0.02% NaN_3 (pH 7.0). Sonicated egg phosphatidylcholine vesicles (containing 0.03 mol % [^{3}H]cholesteryl oleate as a non-exchangeable marker) were prepared in the same buffer. After incubation these vesicles were separated by DEAE-cellulose chromatography. The vesicles prepared from microsomal lipid were completely bound to the column, whereas the egg phosphatidylcholine vesicles were eluted with the incubation buffer from the column with 70% recovery. Exchange was calculated from the ^{32}P/^{3}H ratio in the lipid extract of the eluate.

Mannose 6-phosphatase assay. The barium salt of mannose-6-phosphate (Boehringer, Mannheim) was converted to the sodium salt by addition of an equivalent amount of Na_2SO_4 $BaSO_4$ was centrifuged off. A 10 mM solution of mannose 6-phosphate was made 250 mM with respect to maleic acid and 375 mM with respect to Tris. The pH of the solution was adjusted to 6.3. Aliquots of 0.1 ml of this solution were incubated with 0.1—0.3 mg of microsomal protein at 37°C in a total volume of 1 ml (the mixture contained always 0.25 M sucrose). The reaction was stopped by addtion of 0.1 ml of 50% (w/v) trichloroacetic acid. The precipitate was centrifuged down (10 min at 16 000 × *g*) and the amount of inorganic phosphate in the supernatant was measured according to Chen et al. [13]. From the time course of the reaction (followed

up to 20 min) the initial rate of phosphate release was determined.

Phospholipase A_2 treatment of microsomes. Phospholipase A_2 (*Naja naja*), purified as described by Cremona and Kearny [20], was a gift of Dr. R.F.A. Zwaal (Utrecht). Rat liver microsomes were treated with phospholipase A_2 as described by Nilsson and Dallner [8] under slightly modified conditions (see legends to figures and tables). After stopping the reaction with 5 mM EDTA, the incubation mixture was extracted according to Bligh and Dyer [11]. The aqueous layer was washed twice with chloroform. The lipid extract was then separated by two-dimensional thin-layer chromatography [12]. The spots were detected with iodine vapour and scraped into scintillation vials. 16 ml of toluene (0.5% PPO, 0.03% POPOP)/Triton X-100/water (2 : 1 : 0.2, v/v) was added and ^{32}P radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer. The extent of phosphatidylcholine and phosphatidylethanolamine hydrolysis was calculated from the ratio of the intact phospholipids and their corresponding lyso derivatives.

Determination of microsomal water compartments. Measurements of intra- and extramicrosomal water compartments were carried out essentially according to Nilsson et al. [21]. Approx. 10 mg of microsomal protein was suspended in 5 ml 0.25 M sucrose/20 mM Tris · HCl/0.02% NaN_3 (pH 7.4). A concentrated solution of dextran (purum, M_r 70 000, obtained from Fluka) was added so that the resulting concentration was 15 mg/ml. Furthermore 1 μCi of [^{14}C]-carboxyldextran was added (M_r 70 000, specific activity 1.043 mCi/g, obtained from New England Nuclear). The suspensions were centrifuged for 2 h at 100 000 $\times g$. The insides of the tubes were carefully wiped with filter paper. The pellets were dissolved in 1 ml of formic acid. The density and the total volume of the formic acid solution was determined by weighing. Aliquots (100 μl) of the formic acid solutions were counted in 15 ml dioxane (10% (w/v) naphthalene, 0.7% PPO, 0.03% POPOP) after addition of 100 μl of 0.25 M sucrose buffer. Likewise, 100-μl aliquots of the 100 000 $\times g$ supernatants were counted in the presence of 100 μl formic acid. From these data the dextran accessible space in the microsomal pellets was calculated. Total water content of the pellets was measured by weighing the wet pellets and after drying over H_2SO_4 at 37°C in vacuo till constant weight.

Nuclear magnetic resonance (NMR). ^{31}P-NMR measurements were performed on a Bruker WH 90 spectrometer operating at 36.4 MHz. Proton-decoupled spectra of sonicated phosphatidylcholine vesicles incubated with microsomes were recorded using a sweep width of 1.2 kHz. Maximally 500 transients were accumulated using 90° pulses with an interpulse time 1.7 s. Under these conditions only a narrow spectrum of the vesicles is observed. Peak intensities were determined via computer integration with respect to an external triphenylphosphine reference.

Results

Exchange of microsomal phosphatidylcholine

Phosphatidylcholine exchange protein purified from beef liver was used to exchange microsomal phosphatidylcholine for egg phosphatidylcholine. In these experiments ^{32}P-labeled microsomes were incubated with an excess of egg

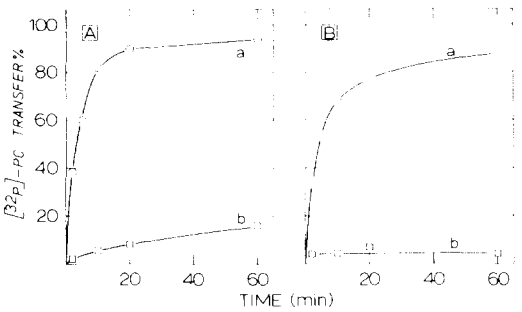

Fig. 1. Exchange of rat liver microsomal [^{32}P]phosphatidylcholine for egg phosphatidylcholine stimulated by phosphatidylcholine exchange protein. (A) Curve a, incubation of 0.54 mg microsomal protein (337 nmol microsomal phosphatidylcholine) with 4400 nmol egg phosphatidylcholine vesicles and 16 μg phosphatidylcholine exchange protein in 150 mM NaCl/1 mM EDTA/10 mM potassium phosphate, pH 7.0 (total volume 1 ml). ^{32}P radioactivity in lipid extract of acceptor vesicles is expressed as percent of [^{32}P]phosphatidylcholine radioactivity originally present in the microsomes. Curve b, control experiment without exchange protein. (B) Microsomes were incubated for 16 h at 4°C in 3% (w/v) glutaraldehyde/150 mM NaCl/1 mM EDTA/10 mM potassium phosphate (pH 7.0). Excess glutaraldehyde was removed by spinning down and resuspending in fresh buffer without glutaraldehyde (three times). Curve a: incubation of glutaraldehyde-treated microsomes (0.48 mg protein, 365 nmol phosphatidylcholine) with 4400 nmol egg phosphatidylcholine vesicles and 16 μg exchange protein. Curve b: control experiment without exchange protein. PC, phosphatidylcholine.

phosphatidylcholine vesicles in order to prevent significant back flow of [^{32}P]-phosphatidylcholine to the microsomes. The experiment shown in Fig. 1A was carried out at 37°C, using a relatively high concentration of exchange protein. Under these conditions the process has a half-time of 2—3 min. An experiment with even a higher ratio of exchange protein to microsomes was not performed, so that it cannot be excluded that the exchange protein was still the rate-limiting factor for the exchange process. In the absence of exchange protein there was also transfer of [^{32}P]phospholipid to the sonicated vesicles, but this was a slow process compared with the protein-mediated phosphatidylcholine exchange. It can be seen that 90—95% of microsomal [^{32}P]phosphatidylcholine is readily transferred to the sonicated vesicles. Assuming that 70% of the egg phosphatidylcholine is available for exchange, is can easily be calculated that practically 100% of microsomal phosphatidylcholine can be exchanged within 1 h at 37°C.

For comparison we measured the exchange of [^{32}P]phosphatidylcholine in sonicated vesicles prepared from total microsomal lipid extract by incubation with sonicated egg phosphatidylcholine vesicles. It can be seen (Fig. 2) that approx. 60% of [^{32}P]phosphatidylcholine is directly available for exchange. This value corresponds to the phosphatidylcholine exchangeability in sonicated rat liver phosphatidylcholine vesicles [5]. Sepharose 2B chromatography of the sonicated microsomal lipids showed only one symmetrical peak of ^{32}P radioactivity in the internal volume of the column, indicating that the preparation consisted of single bilayer vesicles only.

Because of the marked difference in exchangeable phosphatidylcholine pool size when we compared microsomes with sonicated lipid vesicles, we suspected the microsomal membrane structure (i.e. the presence of proteins) to be responsible for the total exchangeability. Therefore we attempted to disturb this

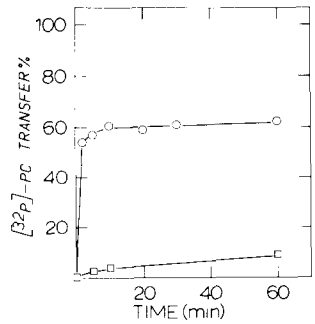

Fig. 2. Phosphatidylcholine exchange between sonicated vesicles prepared from total microsomal lipid and sonicated egg phosphatidylcholine vesicles. Sonicated vesicles from total lipid extract of ^{32}P-labeled rat liver microsomes (98 nmol of phosphatidylcholine) were incubated at 37°C with 1900 nmol of sonicated egg phosphatidylcholine vesicles in a total volume of 1.05 ml. The medium was 12.5 mM potassium phosphate, 1 mM EDTA (pH 6.9). ○———○, incubation with 32 μg of phosphatidylcholine exchange protein; □———□, blank incubation without exchange protein. Transfer of [^{32}P]phosphatidylcholine to the sonicated egg phosphatidylcholine vesicles was measured after separation of the two vesicle populations as described in Materials and Methods. PC, phosphatidylcholine.

structure by cross-linking the microsomal proteins. This was effected by treatment with glutaraldehyde. Glutaraldehyde effectively cross-linked free amino groups since no phosphatidylethanolamine could be detected in the lipid extract after treatment. Fig. 1B shows that this treatment had no significant effect on the rate and extent of microsomal phosphatidylcholine exchange.

Temperature may affect the exchangeable phosphatidylcholine pool size of

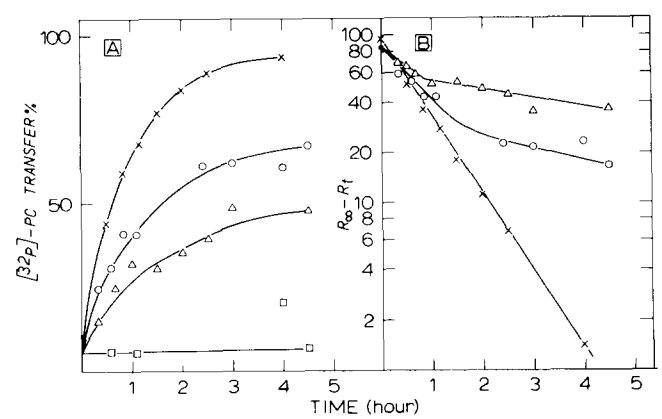

Fig. 3. Temperature effect on phosphatidylcholine exchange between rat liver microsomes and sonicated egg phosphatidylcholine vesicles. (A) Transfer of [^{32}P]phosphatidylcholine from microsomes to sonicated egg phosphatidylcholine vesicles. An aliquot of microsomes containing 91 nmol of phosphatidylcholine was incubated with 660 nmol of egg phosphatidylcholine and 45 μg of exchange protein at 0°C (△———△). An aliquot of microsomes containing 91 nmol of microsomal phosphatidylcholine and 660 nmol of egg phosphatidylcholine were incubated at 8°C with 24 μg of exchange protein (○———○) and without exchange protein (□———□). 150 nmol of microsomal phosphatidylcholine was incubated with 4 μmol of egg phosphatidylcholine in the presence of 9 μg of exchange protein at 25°C (X———X). The medium was 0.15 M NaCl/20 mM Tris · HCl/1 mM EDTA (pH 7.4). (B) Semilogarithmic plot of data shown in A. R_t represents [^{32}P]phosphatidylcholine transfer to the sonicated vesicles (in percent). R_∞ represents [^{32}P]phosphatidylcholine transfer at equilibrium, assuming that only the outer monolayer of the sonicated vesicles and the total microsomal pool are available for exchange.

the microsomes. Exchange experiments were performed at 25, 8 and 0°C, respectively (Fig. 3A). If all microsomal phosphatidylcholine is eventually available for exchange, the [^{32}P]phosphatidylcholine originating from the microsomes will at equilibrium be evenly distributed over the total microsomal pool and the outer monolayer of the sonicated vesicles (70% of total vesicle pool). From these considerations the maximal transfer (R_∞) of [^{32}P]phosphatidylcholine from microsomes to sonicated vesicles can be calculated. If total microsomal phosphatidylcholine behaves as a single pool in the exchange process, a plot of log ($R_\infty - R_t$) against time should result in a straight line. This holds for the exchange process at 25°C, but not at 8 and 0°C (Fig. 3B). This indicates that microsomal phosphatidylcholine acts as a single pool in the exchange process at 25°C, but not at 8 and 0°C.

Phosphatidylcholine exchange and membrane permeability

If phospholipid exchange proteins are used to study transverse movements of phospholipids in membranes, the membrane under study ought to be impermeable to the exchange protein. However, the continuous replacement of phosphatidylcholine molecules via exchange protein might have an effect on the permeability of the membrane. Therefore a permeability test on the microsomes in the presence of exchange protein was carried out.

Mannose-6-phosphatase has been shown to be a sensitive indicator of microsomal membrane integrity [22]. Table I shows that the detergent lysophosphatidylcholine dramatically stimulates microsomal mannose-6-phosphatase activity, whereas phosphatidylcholine exchange protein has very little effect, if any. Therefore, the exchange protein does not increase the membrane's permeability for mannose 6-phosphate, while an active exchange of phosphatidylcholine between the individual microsomal vesicles takes place.

However, in the determination of the exchangeable phosphatidylcholine pool size, microsomal phosphatidylcholine was replaced by egg phosphatidylcholine. To exclude the possibility that the replacement by egg phosphatidylcholine produces leaky microsomes, we measured mannose-6-phosphatase activity of microsomes isolated after incubation with egg phosphatidylcholine vesicles and exchange protein. No increase of enzyme activity was observed after 90% of microsomal phosphatidylcholine had been replaced by egg phosphatidylcholine.

TABLE I

MANNOSE-6-PHOSPHATASE ACTIVITY OF RAT LIVER MICROSOMES

Rat liver microsomes (215 μg protein) were incubated with 1 μmol of mannose-6-phosphatase at 37°C in a total volume of 1 ml. Further details are given in Materials and Methods.

Addition	Activity (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein)
None	8.3
40 nmol lysophosphatidylcholine	85
80 nmol lysophosphatidylcholine	370
4 μg exchange protein	7.6
16 μg exchange protein	16

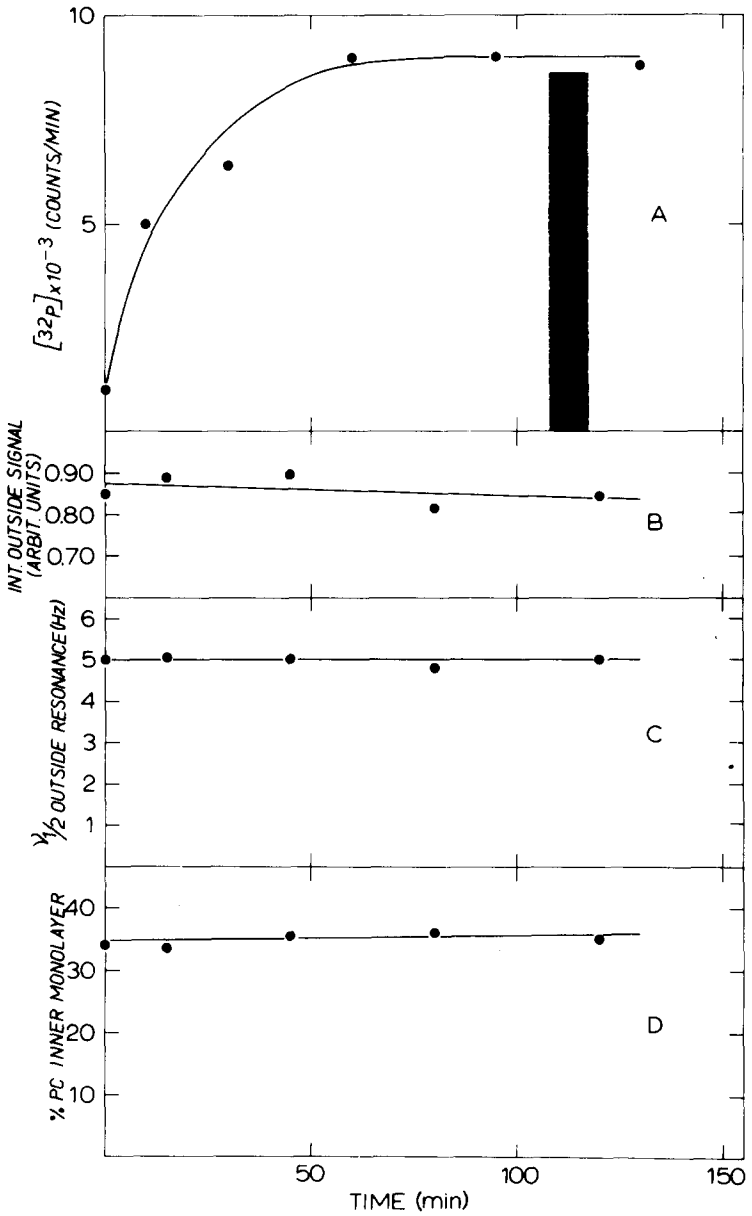

Fig. 4. Integrity of sonicated phosphatidylcholine vesicles during protein catalyzed phosphatidylcholine exchange with rat liver microsomes at 25°C. Vesicles were prepared by sonication of 50 mg (67 μmol) of egg phosphatidylcholine in 1.5 ml of buffer (100 mM NaCl/0.2 mM EDTA/25 mM Tris/acetic acid, pH 7.0) in the presence of 3.3 mM $NdCl_3$. This preparation was dialyzed overnight against the buffer without $NdCl_3$. These vesicles were incubated with phosphatidylcholine exchange protein (63 μg) and ^{32}P-labeled rat liver microsomes (19 mg protein, 4.4 μmol phosphatidylcholine) in a total volume of 4 ml of buffer. The incubation mixture was contained in a NMR tube at 25°C. Only the lower part of the mixture was occupying the space susceptible for ^{31}P NMR measurements. Samples of 250 μl were taken from the mixture at the indicated times for determination of [^{32}P]phospholipid transfer. The meniscus of the mixture was always above the level below which effective NMR measurements could be made. (A) [^{32}P]-Phospholipid recovered from the sonicated vesicles in 250-μl samples. The height of the vertical bar represents the amount of ^{32}P radioactivity of phosphatidylcholine originally present in the microsomes in this volume. (B) Relative intensity of the unshifted signal arising from the phosphatidylcholine molecules in the outer monolayer. (C) Line width of the unshifted signal arising from the phosphatidylcholine molecules in the outer monolayer. (D) Percentage of phosphatidylcholine molecules present in the inner monolayer of the phosphatidylcholine vesicle.

In the following experiment we tested the integrity of the acceptor phosphatidylcholine vesicles during the exchange process. Sonicated egg phosphatidylcholine vesicles loaded with the shift reagent Nd^{3+} were incubated with ^{32}P-labeled microsomes (donor membrane) and phosphatidylcholine exchange protein. Since the shift reagent is present only in the inside of the phosphatidylcholine vesicles, only the inside ^{31}P-NMR signals are shifted, whereas the outside signals are not. At different incubation times the ^{31}P-NMR spectrum was recorded and samples were taken to measure the extent of [^{32}P]phosphatidylcholine transfer. After 1 h phosphatidylcholine exchange was practically complete (Fig. 4A) but no change in the intensity (Fig. 4B) and line width (Fig. 4C) of the unshifted resonance was observed. Moreover, the percentage of phosphatidylcholine molecules present in the inner monolayer remained constant (Fig. 4D) and the position of the shifted inside resonance did not change during the exchange process. This indicates that during phosphatidylcholine exchange the Nd^{3+} did not leak out of the vesicles. Furthermore, under these conditions association of the vesicles with the microsomes was not detected, since this would result in a loss of signal intensity and/or broadening of the spectral peak. No such effects were observed. After 20 h incubation microsomes and vesicles were separated and the vesicle lipids were analyzed by two-dimensional thin-layer chromatography. Besides in minor amounts of phosphatidylethanolamine and sphingomyelin the bulk of ^{32}P radioactivity (94%) was located in the phosphatidylcholine spot.

Phospholipase A_2 treatment of rat liver microsomes

The complete availability of microsomal [^{32}P]phosphatidylcholine for exchange at 37 and 25°C is in contrast to the limited hydrolysis catalyzed by *N. naja* phospholipase A_2 at 0 and 30°C as reported by Nilsson and Dallner [8,9]. These investigators found that at 0°C only 55% of the microsomal phosphatidylcholine pool could be hydrolyzed provided that high concentrations of bovine serum albumin were present [8]. At 30°C 40% of microsomal phosphatidylcholine was readily hydrolyzed and the remaining of this lipid only after prolonged incubation [9]. We repeated these experiments not only at 0°C but also at 37°C, the temperatures at which part of the exchange experiments were carried out. Fig. 5 shows that our experiments at 0°C are in reasonable agreement with those of Nilsson and Dallner, although a second dose of phospholipase A_2 after 110 min increases the hydrolysis up to 61%. At 37°C phosphatidylcholine hydrolysis proceeds to 67% and a second dose of enzyme after 110 min results in 80% hydrolysis. Phosphatidylethanolamine hydrolysis at 0 and 37°C reaches 87 and 96%, respectively. It should be noted that the calculation of phospholipid hydrolysis from ^{32}P radioactivity measurements is allowed, since the specific radioactivities of hydrolyzed and non-hydrolyzed phospholipids are the same [8].

According to Nilsson and Dallner the intramicrosomal space is not accessible for high molecular weight dextran after phospholipase A_2 treatment at 0°C [8] and at 30°C [9] provided that high concentrations of albumin are present. We performed similar measurements for microsomes treated with phospholipase A_2 at 37°C (Table II). Phospholipase treatment in the absence of albumin results in a drastic reduction of dextran-inaccessible space. Only a minor

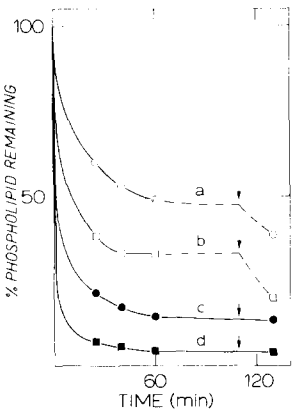

Fig. 5. Phospholipase A_2 (*N. naja*) treatment of ^{32}P-labeled rat liver microsomes. Microsomes (9 mg protein) were incubated with 235 mg bovine serum albumin and 6.5 units of phospholipase A_2 in 20 mM Tris · HCl buffer (pH 7.2), containing 0.25 M sucrose and 0.02% NaN_3 (total volume 5 ml). Curve a, phosphatidylcholine (incubation temperature 0°C); curve b, phosphatidylcholine (incubation temperature 37°C); curve c, phosphatidylethanolamine (incubation temperature 0°C); curve d, phosphatidylethanolamine (incubation temperature 37°C). After 110 min incubation an additional amount of phospholipase A_2 (6.5 units) was added (arrows).

decrease of intramicrosomal water is observed after phospholipase treatment in the presence of 5% (w/v) bovine serum albumin.

Fatty acids and lysophospholipids may be inhibitory to the phospholipase A_2 action and thus account for the limited hydrolysis of microsomal phospholipids. Therefore we separated the membranes after phospholipase treatment from the bulk of the hydrolysis products (bound to bovine serum albumin) and reincubated the membranes with a fresh amount of phospholipase A_2. Phosphatidylcholine hydrolysis proceeded with a slow rate. After 2 h at 37°C about 50% of phosphatidylcholine present at the beginning of the second incubation was still remaining.

Apparently, two pools of microsomal phosphatidylcholine (and phosphatidylethanolamine) exist: one pool directly available for phospholipase A_2 attack, and a second pool which is only very slowly degradable. Do these pools have a different composition in terms of molecular species of phosphatidyl-

TABLE II

MICROSOMAL WATER COMPARTMENTS AFTER PHOSPHOLIPASE A_2 TREATMENT

Phospholipase A_2 (*N. naja*) (units/mg microsomal protein)	Albumin/ microsomal protein (mg/mg)	Incubation		Dextran space (μl/mg dry wt.)	Total H_2O (μl/mg dry wt.)	Intramicrosomal H_2O/total H_2O (%)
		Temperature (°C)	Time (min)			
0.7	25	37	60	2.3	3.9	42
0.7	0	37	60	3.3	4.5	27
0.7	25	0	60	1.9	3.4	43
0	25	37	60	1.7	3.5	51
0	0	37	60	1.8	3.7	51

TABLE III

FATTY ACID COMPOSITION OF MICROSOMAL PHOSPHATIDYLCHOLINE BEFORE AND AFTER PHOSPHOLIPASE A_2 TREATMENT

Phospholipase A_2	Incubation temperature (°C)	Hydrolysis (%)	Fatty acid (percent of total) *				
			16 : 0	18 : 0	18 : 1	18 : 2	20 : 4
—	—	—	28	30	7	12	24
+ **	0	61	32	34	8	11	15
+ **	37	80	34	36	7	8	15

* Fatty acid compositions were calculated from the peak areas of the gas chromatograms.

** Microsomes were incubated for 130 min with phospholipase A_2 under the same conditions as in Fig. 3.

choline? The fatty acid composition of this phospholipid was determined before and after phospholipase treatment. Table III shows that the content of arachidonic acid has decreased after phospholipase A_2 treatment both at 0°C and at 37°C.

One may wonder whether the phosphatidylcholine remaining after phospholipase A_2 treatment is still available for exchange protein. ^{32}P-labeled rat liver microsomes were incubated with phospholipase A_2 at 37°C, which resulted in 64% hydrolysis of [^{32}P]phosphatidylcholine. After removal of the bulk of the lysophospholipids (bound to bovine serum albumin), the microsomes were incubated with egg phosphatidylcholine vesicles and phosphatidylcholine exchange protein. After 2 h almost all microsomal [^{32}P]phosphatidylcholine had been transferrred to the sonicated vesicles. It may be of importance to note that the time required for complete transfer of microsomal [^{32}P]phosphatidylcholine remaining after phospholipase A_2 treatment is much larger than in a comparable experiment with intact microsomes (experiment not shown).

Discussion

Rat liver microsomal phosphatidylcholine can be completely replaced by egg phosphatidylcholine from sonicated vesicles in the presence of phosphatidylcholine exchange protein from beef liver. Assuming that the microsomal phospholipids are organized in a bilayer structure this observation can be explained in two different ways. Firstly, all phosphatidylcholine is present in the cytoplasmic leaflet of the membrane. Alternatively, phosphatidylcholine is present in both luminal and cytoplasmic halves of the bilayer, but rapid transbilayer movement of phosphatidylcholine causes complete mixing of the spatially separated pools. Permeation of exchange protein through the microsomal membrane during the exchange process can be excluded since permeability to a small molecular weight solute (mannose 6-phosphate) was also low. The complete exchangeability of phosphatidylcholine in rat liver microsomes is in contrast to the limited exchangeability in sonicated vesicles prepared from the extracted lipids. In the latter system apparently only the outer monolayer is available for exchange. Similar observations were made for mitochondria from which the outer membrane was removed. In this case the phosphatidylcholine was completely available for exchange, whereas in sonicated vesicles derived

from mitochondrial lipids the phosphatidylcholine could only be exchanged for 65% (data not shown). Induction of transmembrane movement in microsomes by the exchange process cannot be excluded, although it does not occur in sonicated vesicles prepared from microsomal lipids. The possibility that complete exchangeability of microsomal phosphatidylcholine was induced by replacement with different molecular species of egg phosphatidylcholine was made unlikely by showing that incubation of microsomes with an excess of vesicles prepared from rat liver phosphatidylcholine resulted also in complete exchange of microsomal phosphatidylcholine. Microsomal proteins might be involved in the phenomenon of complete phosphatidylcholine exchangeability. However, cross-linking of the microsomal amino groups with glutaraldehyde does not have much of an effect on phosphatidylcholine exchange. At lower temperatures (8 and 0° C) microsomal phosphatidylcholine is not exchanged as a single pool. At 25 and 37° C probably rapid equilibrium between the pools takes place. It is not yet possible to localize these pools spatially.

Phospholipases have been used extensively for the localization of phospholipids in biological membranes. This method has the disadvantage that the membrane structure is disturbed or even disrupted. Nilsson and Dallner [8] found that phospholipase A_2 treatment of rat liver microsomes at 0° C results in 55% hydrolysis of total phospholipids while the permeability of the membrane to macromolecules is not increased. Only 55% of phosphatidylcholine could be hydrolyzed under their conditions. In the present study 60% of phosphatidylcholine could be hydrolyzed at 0° C, but 80% at 37° C. If the microsomal phospholipids which are readily available for hydrolysis, represent the cytoplasmic leaflet of the membrane, it is not clear why the degree of hydrolysis depends on the temperature of incubation. Furthermore, this interpretation implies slow transmembrane movement of phospholipid during the hydrolysis process. However, Nilsson and Dallner [8] found that after phospholipase A_2 treatment of microsomes, obtained only 1 h after injection of [^{32}P]phosphate in the rat, the remaining intact phosphatidylcholine had the same specific radioactivity as the hydrolyzed phosphatidylcholine. This indicates that equilibrium was established between the different phosphatidylcholine pools. In this respect it is worthwhile to mention the study by Bevers et al. [23], who found that the extent of phosphatidylglycerol hydrolysis in *Acholeplasma laidlawii* cells and isolated membranes by phospholipase A_2 was highly dependent on the temperature. Not only the packing of the lipid molecules, but also protection by other membrane constituents were suggested to be responsible for this phenomenon.

Apparently, a paradox exists between the interpretations of the results of the exchange experiments and phospholipase A_2 treatments. Two questions have to be answered. (1) If transmembrane movement in microsomes is slow and only part of phosphatidylcholine is in the cytoplasmic leaflet, why can all phosphatidylcholine be exchanged? The possibility of induction of transmembrane movement by the exchange process has been discussed previously. (2) If rapid transmembrane movement of phosphatidylcholine occurs, why can only part of it be hydrolyzed? One possible explanation may be that degradation of more than 50% of the membrane phospholipids results in a drastic change of membrane structure with a concomitant loss of the facility of

transmembrane movement of phosphatidylcholine. This interpretation is supported by the fact that the phosphatidylcholine exchange rate of the remaining lipid after phospholipase treatment is decreased when compared with the intact microsomes. A second possibility would be that the phospholipids in the microsomal membrane are in different states of aggregation, which have a different susceptibility for phospholipase A_2 attack. The existence of different lipid domains in the microsomal membrane has been suggested by several authors [24,25]. Our results on the different fatty acid composition of readily and slowly hydrolyzable phosphatidylcholine pools in the microsomes is in line with this idea of lipid segregation in the membrane.

Transbilayer movement of phosphatidylcholine in erythrocyte membranes has been demonstrated by several investigators [26,27], the half-time of this process being a few hours. From a physiological point of view, microsomal membranes may be expected to allow considerable rates of phospholipid transmembrane movements. The liver endoplasmic reticulum is the site of synthesis of several phospholipids and serum lipoproteins [28,29]. Since the biosynthesis of phosphatidylcholine has recently been shown to be localized on the cytoplasmic side of the membrane [30,31], transport mechanisms for the product have to exist, since secretion occurs via the lumen of this membrane system [7].

At this state, it is difficult to decide between rapid and slow transmembrane movement of phosphatidylcholine in liver microsomes since the initial transverse distribution of the phospholipids in this membrane is not known with certainty. The original results of Nilsson and Dallnner [8,9] have recently been questioned by Sundler et al. [32], although both groups arrive at the conclusion of an almost equal distribution of the phosphatidylcholine over the cytoplasmic and cisternal half of the membrane. Yet, another distribution has been reported by Higgins and Dawson [33]. These investigators concluded that 75% of the phosphatidylcholine was located in the outer monolayer of isolated microsomal membranes. The rapid exchangeability of at least 90% of the phosphatidylcholine as reported here would then seem to indicate a rapid transbilayer movement of at least part of the phosphatidylcholine pool. When this work was in preparation, Zilversmit and Hughes [34] arrived at similar conclusions on the exchangeability of phosphatidylcholine in rat liver microsomes. However, the half-time for the exchange of phosphatidylcholine in our experiments was at least 10-fold smaller than reported by Zilversmit and Hughes [34].

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References

1 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 702—731
2 Devaux, P. and McConnell, H.M. (1972) J. Am. Chem. Soc. 94, 4475—4481

3 Cullis, P.R. (1976) FEBS Lett. 70, 223—228
4 Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809—2816
5 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
6 de Kruijff, B. and Wirtz, K.W.A. (1977) Biochim. Biophys. Acta 468, 318—326
7 Rothman, J.E. and Lenard, J. (1977) Science 195, 743—753
8 Nilsson, O. and Dallner, G. (1977) J. Cell Biol. 72, 568—583
9 Nilsson, O. and Dallner, G. (1975) FEBS Lett. 58, 190—193
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
12 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457—461
13 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
14 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
15 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600—608
16 Papahadjopoulos, D. and Miller, N. (1967) Biochim. Biophys. Acta 135, 624—638
17 van den Besselaar, A.M.H.P., Helmkamp, G.M. and Wirtz, K.W.A. (1975) Biochemistry 14, 1852—1858
18 Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry 16, 2806—2810
19 Kamp, H.H., Wirtz, K.W.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 318, 313—325
20 Cremona, T. and Kearny, E.B. (1963) J. Biol. Chem. 239, 2328—2334
21 Nilsson, R., Peterson, E. and Dallner, G. (1973) J. Cell Biol. 56, 762—776
22 Arion, A.J., Ballas, L.M., Lange, A.J. and Wallin, B.K. (1976) J. Biol. Chem. 251, 4901—4907
23 Bevers, E.M., Singal, S.A., Op den Kamp, J.A.F. and van Deenen, L.L.M. (1977) Biochemistry 16, 1290—1295
24 Stier, A. and Sackmann, E. (1973) Biochim. Biophys. Acta 311, 300—408
25 Mabrey, S., Powis, G., Schenkman, J.B. and Tritton, T.R. (1977) J. Biol. Chem. 252, 2929—2933
26 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277—1283
27 Renooy, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58
28 Polonovski, J. (1976) Biochimie 58, 971—980
29 Schumaker, V.N. and Adams, G.H. (1969) Annu. Rev. Biochem. 38, 113—136
30 Vance, D.E., Choy, P.C., Farren, S.B., Lim, P.H. and Schneider, W.J. (1977) Nature 270, 268—269
31 Coleman, R. and Bell, R.M. (1978) J. Cell Biol. 76, 245—253
32 Sundler, R., Sarcione, S.L., Alberts, A.W. and Vagelos, P.R. (1977) Proc. Natl. Acad. Sci. U.S. 74, 3350—3354
33 Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342—356
34 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110