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INTERACTION OF PHOSPHATIDYLCHOLINE LIPOSOMES AND PLASMA LIPOPROTEINS WITH SHEEP ERYTHROCYTE MEMBRANES

PREFERENTIAL TRANSFER OF PHOSPHATIDYLCHOLINE CONTAINING UNSATURATED FATTY ACIDS

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The interaction of sheep erythrocyte membranes with phosphatidylcholine vesicles (liposomes) or human plasma lipoproteins is described. Isolated sheep red cell membranes were incubated with liposomes containing [^{14}C]phosphatidylcholine or [^{3}H]phosphatidylcholine in the presence of EDTA. A time-dependent uptake of phosphatidylcholine into the membranes could be observed. The content of this phospholipid was increased from 2 to 5%. The rate of transfer was dependent on temperature, the amount of phosphatidylcholine present in the incubation mixture and on the fatty acid composition of the liposomal phosphatidylcholine. A possible adsorption of lipid vesicles to the membranes could be monitored by adding cholesteryl [^{14}C]oleate to the liposomal preparation. As cholesterylesters are not transferred between membranes [1], it was possible to differentiate between transfer of phosphatidylcholine molecules from the liposomes into the membranes and adsorption of liposomes to the membranes. The phosphatidylcholine incorporated in the membranes was isolated, and its fatty acids were analysed by gas chromatography. It could be shown that there was a preferential transfer of phosphatidylcholine molecules containing two unsaturated fatty acids.

Introduction

Lipid exchange between plasma lipoproteins and surface membranes of most cells is of great importance for the maintenance of the composition and asymmetry of the plasma membrane lipids. Studies on red cells have shown the differential exchange rates of cholesterol and the various phospholipids [2].

As red blood cells cannot synthesize sterols or fatty acids, they solely depend on exchange processes for the maintaining of the integrity of their membrane lipids. A limited remodelling of membrane phospholipids is realized by acyl transferases present in the red cell membrane [3].

In contrast to most of the other erythrocyte membranes, those from ruminants, especially sheep, only contain traces of phosphatidylcholine [4]. This phenomenon is difficult to understand because ruminant plasma lipoproteins contain equally high amounts of phosphatidylcholine (approx. 70%) as those from most of the other species. Ruminant erythrocyte membranes contain an externally located membrane-bound phospholipase A_2 with preference for phosphatidylcholine [5]. This enzyme is responsible for the low phosphatidylcholine content in those membranes. As could be shown, the resulting split products, i.e. free fatty acids and lyso-compounds, are released to the plasma lipoprotein or albumin (Rindlisbacher, B. and Zahler, P., unpublished data).

Transfer and accumulation of phosphati-

dylcholine in sheep red cell membranes can be studied in the presence of EDTA. Preliminary observations with radiolabelled compounds indicate that the transfer rate is dependent upon the fatty acid composition of the phospholipid. In the present work this question is studied in more detail using both liposomes and plasma lipoproteins as lipid donor.

Experimental procedures

Materials. Egg yolk phosphatidylcholine was obtained from Lipid Products, Nuthill, U.K. Dipalmitoyl phosphatidylcholine was from Fluka. [U-^{14}C]phosphatidylcholine (from *Chlorella pyrenoidosa*) (18 Ci/mmol), [1-^{14}C]dipalmitoylphosphatidylcholine (100 mCi/mmol) and cholesteryl [1-^{14}C]oleate (51 mCi/mmol) were purchased from New England Nuclear.

Egg-[*Me*-^{3}H]phosphatidylcholine was prepared as described by Stoffel et al. [7] using [^{3}H]methyliodide (1.25 Ci/mmol) from Amersham International, U.K.

All lipid preparations were periodically checked for degradation products and, if necessary, repurified by thin-layer chromatography on silicagel 60, 0.25 mm, from Merck.

Solvents used for lipid extraction and thin-layer chromatography were freshly distilled.

Erythrocyte membranes were isolated according to Dodge et al. [8]. Pooled human serum was obtained from the Swiss National Blood Transfusion Service of the Red Cross.

Methods. Radioactive and nonradioactive phosphatidylcholine were mixed in chloroform/methanol (1 : 1, by vol.). In certain experiments, trace amounts of cholesteryl [^{14}C[oleate were added to the mixture. The solvent was evaporated under nitrogen. The lipids were suspended in 10 mM glycylglycine buffer (pH 8.0) at a concentration of 10 mg phosphatidylcholine/ml. Liposomes were prepared by sonication as described by Shinitzky et al. [9].

Human serum was incubated at 58°C for 30 min to inactivate the lecithin: cholesterol acyltransferase. Serum lipoproteins were labelled by incubation of whole serum with [^{3}H]phosphatidylcholine containing liposomes at 37°C for 30 min. High density lipoproteins (HDL) were isolated by density gradient centrifugation according to Redgrave et al. [10].

Equal volumes of red cell membranes (5–10 mg protein/ml glycylglycine buffer, 10 mM, pH 8.0) and liposomes, whole serum or isolated HDL were incubated at 37°C under constant stirring. The mixture contained 1 mM EDTA. Repeatedly, aliquots were taken, centrifuged at $12\,000 \times g$ for 20 min to separate the membranes from the liposomes or serum lipoproteins. The membranes were washed three times with 10 mM glycylglycine buffer (pH 8.0), 1 mM EDTA. The radioactivity incorporated per mg protein was determined. Radioactivity was measured by liquid scintillation counting. Protein was determined according to Lowry et al. [11].

For gas chromatographic analysis of the transferred phosphatidylcholine the membranes were incubated with the liposomes or human serum at 37°C during 3 h. After the incubation the lipids were extracted according to the method of Renkonen [12]. The lipids were separated by thin-layer chromatography on silicagel using the solvent system: chloroform/methanol/acetic acid/water (65 : 35 : 15 : 4, by vol.). Phosphatidylcholine was extracted from the silicagel with chloroform/methanol (2 : 1, by vol.). The fatty acids were esterified according to Thies [13] and the esterified fatty acids were analysed on a Perkin Elmer Sigma Gaschromatograph by isothermic separation at 190°C using a capillary column WCOT, packed with Carbowax 20 M.

Results

Phosphatidylcholine uptake from liposomes. Fig. 1 shows the appearance of radioactively-labelled PC in sheep red cell membranes during the incubation with liposomes containing [^{14}C]phosphatidylcholine. In the presence of EDTA an increase of the radioactive lipid in the membranes could be observed. Contrastingly, in the presence of Ca^{2+} [^{14}C]phosphatidylcholine appeared more slowly and levelled off after about 200 min. Thus, in both cases phosphatidylcholine was taken up from liposomes into the membranes. In the presence of Ca^{2+} the membraneous phospholipase A_2 is active and hydrolyses the transferred phosphatidylcholine to lysophosphatidylcholine and free fatty acids [14].

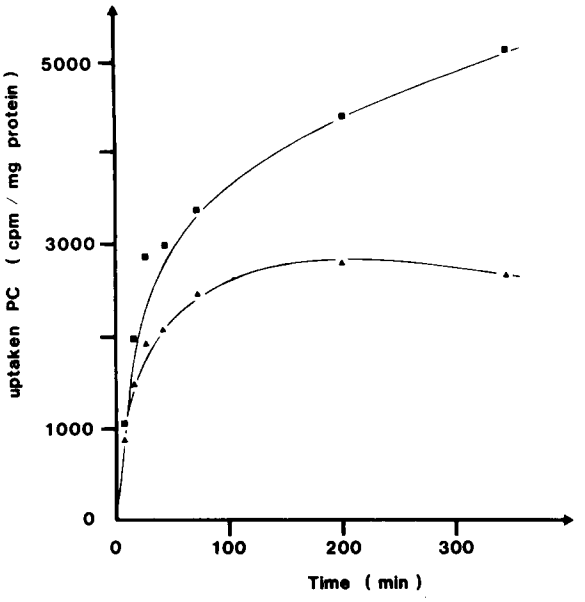

Fig. 1. Uptake of [^{14}C]phosphatidylcholine from liposomes into sheep erythrocyte membranes. 10 ml phosphatidylcholine liposomes (40 mg egg phosphatidylcholine $+5 \cdot 10^5$ cpm [^{14}C]phosphatidylcholine from *C. pyrenoidosa* in 10 ml glycylglycine buffer, 10 mM, pH 8.0) were incubated with 10 ml sheep red cell membranes (6.4 mg protein/ml) at 37°C as described. The incubation mixture contained either 1 mM EDTA (■———■) or 1 mM Ca^{2+} (▲———▲).

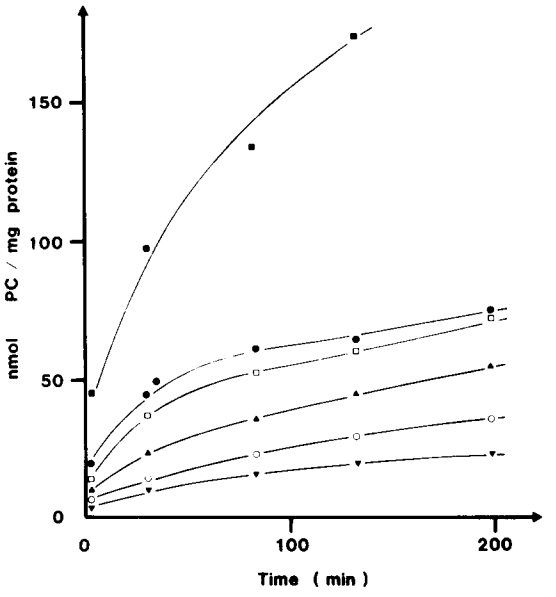

Fig. 2. Temperature dependence of phosphatidylcholine uptake into sheep erythrocyte membranes. 2.5 ml liposomes (5 mg egg phosphatidylcholine $+4 \cdot 10^5$ cpm [^{14}C]phosphatidylcholine from *C. pyrenoidosa* in 2.5 ml 10 mM glycylglycine buffer, pH 8.0) were incubated with 2.5 ml sheep red cell membranes (6.4 mg protein/ml) at the above temperatures in the presence of 1 mM EDTA. The uptaken phosphatidylcholine was determined as described. ■, 50°C; ●, 40°C; □, 30°C; ▲, 20°C; ○, 10°C; ▼, 0°C.

It could be shown that the rate of transfer is dependent on the temperature (Fig. 2) and on the amount of phosphatidylcholine present in the incubation mixture (results not shown).

Control of the adsorption of liposomes on the membranes. In order to exclude that the observed increase of [^{14}C]phosphatidylcholine in the membrane fraction is due to an unspecific adsorption of lipid vesicles on the membranes or fusion, trace amounts of cholesteryl [^{14}C]oleate were added to the liposomal preparation. Cholesteryl esters are not exchanged between membranes [1]. Therefore, any radioactivity found in the membranes would be attributed to adsorbed vesicles or to a fusion of liposomes with the membranes. Fig. 3 shows the small increase of cholesteryl [^{14}C]oleate in the membrane fraction compared to the increase of [^{14}C]phosphatidylcholine. The experiment was made in two parallel incubations using two different liposome preparations: (a) egg phosphatidylcholine labelled with [^{14}C]phosphatidylcholine from *Chlorella pyrenoidosa* and (b) egg phosphatidylcholine labelled with cholesteryl [^{14}C]-oleate. For both isotopes, [^{14}C]phosphatidylcholine and cholesteryl [^{14}C]oleate, cpm/nmol phosphatidylcholine in the liposomes was determined.

A slight increase of cholesteryl [^{14}C]oleate could be observed as a result of adsorption, fusion or transfer. Compared to the one order of magnitude higher uptake of phosphatidylcholine, the effect of liposome adsorption or fusion with the membrane preparation was minimal.

Phosphatidylcholine uptake from plasma lipoproteins. The same experiments were carried out using human plasma high density lipoproteins containing radioactive phosphatidylcholine as the lipid donor instead of liposomes. Fig. 4 shows that there was a transfer of [^{3}H]phosphatidylcholine from the lipoproteins into the sheep red cell membranes. Adsorption could be neglected, as less than 10% cholesteryl [^{14}C]oleate, compared to the uptake of [^{3}H]phosphatidylcholine, was found in the membranes after the incubation.

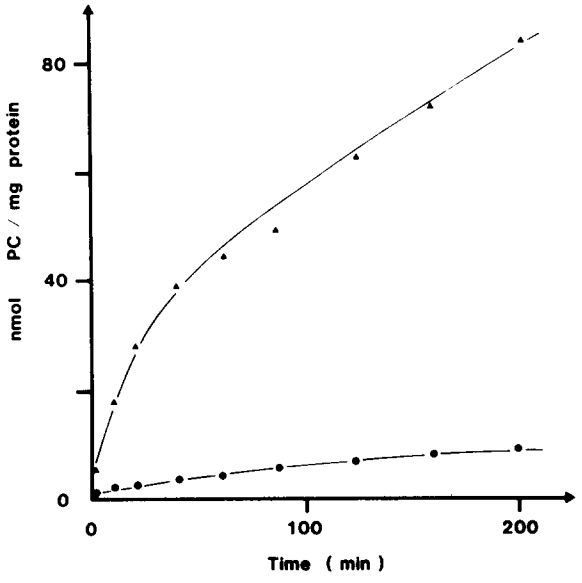

Fig. 3. Increase of cholesteryl [^{14}C]oleate in the membrane compared to [^{14}C]phosphatidylcholine from *C. pyrenoidosa*. 5 ml sheep red cell membranes (6.2 mg protein/ml) were incubated with 5 ml liposomes made from 5 mg egg phosphatidylcholine in 5 ml 10 mM glycylglycine buffer, pH 8.0, labelled with (a) $2 \cdot 10^5$ cpm [^{14}C]phosphatidylcholine from *C. pyrenoidosa* (▲———▲) and (b) $4 \cdot 10^5$ cpm cholesteryl [^{14}C]oleate (●———●) at 37°C as described. The values were calculated as described in Results.

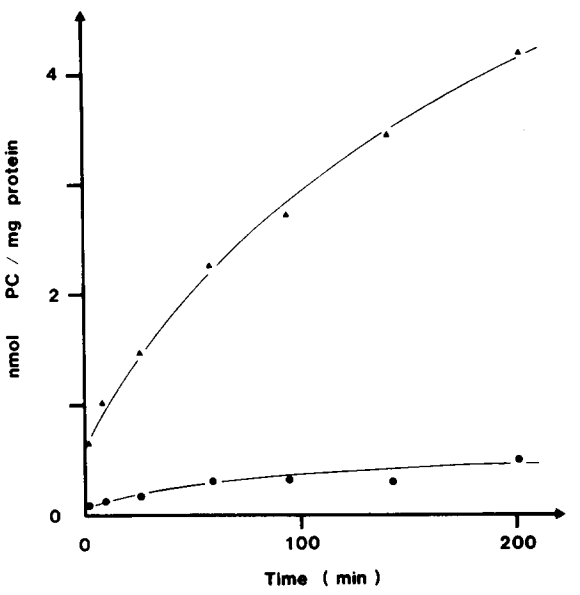

Fig. 4. Uptake of phosphatidylcholine from high density lipoproteins (HDL) into sheep red cell membranes. 4 ml sheep erythrocyte membranes (6.4 mg protein/ml) were incubated with 5 ml HDL (1.5 mg phospholipids/ml) labelled with (a) $6 \cdot 10^5$ cpm egg-[^{3}H]phosphatidylcholine (▲———▲) and (b) $3 \cdot 10^5$ cpm cholesteryl [^{14}C]oleate (●———●) at 37°C in the presence of 1 mM EDTA. The uptake of the radioactive label was determined as described.

Comparison of the transfer rates of phosphatidylcholines from different sources. In the first experiments liposomes prepared from egg phosphatidylcholine labelled with [^{14}C]phosphatidylcholine from *C. pyrenoidosa* were used. Analysis of the specific activity of phosphatidylcholine in the liposomes and in the membranes after the incubation resulted in an up to 10-times higher specific activity in the membranes. This led to the conclusion that for some reason the rate of transfer for [^{14}C]phosphatidylcholine from *C. pyrenoidosa* was higher than for egg phosphatidylcholine. Therefore, it was necessary to use ^{3}H-labelled egg phosphatidylcholine rather than [^{14}C]phosphatidylcholine from *C. pyrenoidosa*, in order to correlate the transferred radioactivity with the real transfer of phosphatidylcholine. Three different preparations of liposomes were prepared: (a) 10 mg egg phosphatidylcholine $+6 \cdot 10^5$ cpm [^{14}C]phosphatidylcholine from *C. pyrenoidosa*, (b) 10 mg egg phosphatidylcholine $+6 \cdot 10^5$ cpm egg-[^{3}H]phosphatidylcholine and (c) 10 mg egg phosphatidylcholine $+7 \cdot 10^5$ cpm [^{14}C]dipalmitoylphosphatidylcholine. Fig. 5 shows the uptake of the different labels into sheep red cell membranes.

The data confirmed that the rate of transfer for [^{14}C]phosphatidylcholine from *C. pyrenoidosa* was higher than that for egg phosphatidylcholine. Egg phosphatidylcholine on the other hand was taken up faster than dipalmitoylphosphatidylcholine.

Basing on these results, it was supposed that the fatty acid composition was decisive for the rate of transfer. Dipalmitoylphosphatidylcholine, which has two saturated fatty acids, was taken up extremely slowly, compared to egg phosphatidylcholine and [^{14}C]phosphatidylcholine from *C. pyrenoidosa*. Egg phosphatidylcholine has a known heterogeneous fatty acid composition, whereas the composition of the purchased [^{14}C]phosphatidylcholine from *C. pyrenoidosa* is not known. However, Hitchcock and Nichols [15] found a fatty acid composition of *C. pyrenoidosa* which is characterized by a high concentration of un-

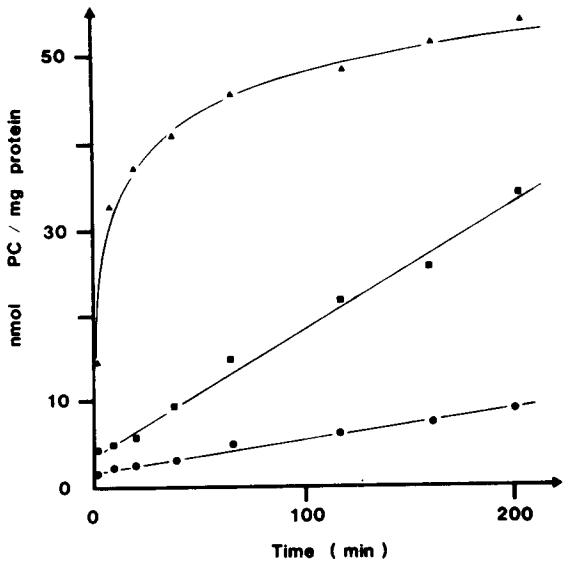

Fig. 5. Uptake of three different phosphatidylcholines from liposomes into sheep erythrocyte membranes. 4 ml sheep red cell membranes (6.2 mg protein/ml) were incubated with 4 ml liposomes made from 10 mg egg phosphatidylcholine in 10 ml 10 mM glycylglycine buffer, pH 8.0, labelled with (a) $6 \cdot 10^5$ cpm [^{14}C]phosphatidylcholine from *C. pyrenoidosa* (▲———▲), (b) $6 \cdot 10^5$ cpm egg-[^{3}H]phosphatidylcholine (■———■) and (c) $7 \cdot 10^5$ cpm [^{14}C]dipalmitoylphosphatidylcholine (●———●) at 37°C in the presence of 1 mM EDTA as described.

saturated fatty acids. This is in agreement with a high transfer activity of phosphatidylcholine molecules.

Fatty acid composition of the transferred phosphatidylcholine. On the basis of the results presented above it was proposed that phosphatidylcholine containing polyunsaturated fatty acids were preferentially transferred. To confirm this working hypothesis, the fatty acid composition of the phosphatidylcholine donor and that of the phosphatidylcholine in the acceptor membrane before and after the transfer experiment were analysed by gas-chromatography. Egg phosphatidylcholine liposomes or whole serum were used as phosphatidylcholine donors.

Tables I and II show the results of these experiments. Egg phosphatidylcholine as well as serum phosphatidylcholine have a highly heterogeneous fatty acid composition. Total hydrolysis of egg phosphatidylcholine with pancreatic phospholipase A_2 (unpublished data) proved that the saturated fatty acids were predominantly found in position 1 of the phosphatidylcholine molecule, whereas position 2 almost exclusively consisted of unsaturated fatty acids. About 8% only of the egg phosphatidylcholine molecules and about 16% of the serum phosphatidylcholine molecules contained two unsaturated fatty acids.

Membraneous phosphatidylcholine was isolated

TABLE I

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE TRANSFERRED FROM LIPOSOMES INTO SHEEP ERYTHROCYTE MEMBRANES

Lipid was extracted from 20 ml sheep red cell membrane suspension (6.2 mg protein/ml) and phosphatidylcholine was isolated as described in the text. The transfer was carried out with 20 ml liposomes (120 mg egg phosphatidylcholine in 20 ml 10 mM glycylglycine buffer, pH 8.0) and 20 ml sheep red cell membranes (6.2 mg protein/ml) as described. The composition of the transferred phosphatidylcholine was calculated, considering that the phosphatidylcholine content in the membranes increased from 3 to 5% of total phospholipid during the incubation.

Chain length	Egg-PC (%)	PC-membranes (%)	PC-membranes after transfer (%)	Transferred PC (%)
16:0	35.5	2.7	2.2	1.4
16: unsaturated	0.3	20.2	19.6	17.1
18:0	10.4	5.0	1.6	
18:1	29.3	6.4	2.1	
18:2	14.6	2.2	0.7	
18:3-20:3	2.7	32.8	20.2	3.4
20:4	3.0	5.9	9.6	13.8
20: > 4	4.2	10.9	18.6	27.5
22: unsaturated	—	13.4	24.6	36.5

TABLE II

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE TRANSFERRED FROM HUMAN SERUM LIPOPROTEINS INTO SHEEP ERYTHROCYTE MEMBRANES

Lipid was extracted from 5 ml human serum and from 20 ml sheep red cell membranes (6.2 mg protein/ml) and phosphatidylcholine was isolated as described. The transfer was carried out with 20 ml sheep erythrocyte membranes (6.2 mg protein/ml) and 30 ml inactivated human serum at 37°C as described. The composition of the transferred phosphatidylcholine was calculated, considering that the phosphatidylcholine content in the membranes increased from 3 to 5% of total phospholipids during the incubation.

Chain length	Serum-PC (%)	PC-membranes (%)	PC-membranes after transfer (%)	Transferred PC (%)
16:0	25.8	2.7	2.4	1.8
16: unsaturated	1.7	20.2	23.1	24.4
18:0	15.6	5.0	1.7	
18:1	10.7	6.4	2.2	
18:2	16.8	2.2	3.6	5.0
18:3–20:3	8.9	32.8	26.4	15.0
20:4	5.9	5.9	6.4	6.4
20: > 4	9.7	10.9	9.5	6.7
22: unsaturated	4.8	13.4	26.4	40.7

before and after the incubation of the sheep red cell membranes with liposomes or human serum. Before the incubation the phosphatidylcholine content amounted to 3%, after the incubation to 5% of the total phospholipids.

Phosphatidylcholine isolated from sheep erythrocyte membranes before the incubation contained few saturated fatty acids. The content of oleic and linoleic acid was very low, too. The main components were polyunsaturated fatty acids like 16: X, 18: X, 20: X and 22: X (where $X > 2$).

The difference in the content of the various fatty acids before and after the incubation was calculated. There was an increase of the polyunsaturated fatty acids, whereas the content of palmitic, stearic, oleic and linoleic acids was not altered. Thus, mainly phosphatidylcholine molecules containing polyunsaturated fatty acids were transferred into sheep red cell membranes, although the offered phosphatidylcholine (egg phosphatidylcholine, resp. human serum phosphatidylcholine) contained mostly palmitic, stearic, oleic and linoleic acids.

Up to 2% of the liposomal phosphatidylcholine was taken up into the membranes during 3 h incubation. Considering that only 8% of the egg phosphatidylcholine contained two unsaturated fatty acids, about 1/4 of those molecules were transferred into sheep erythrocyte membranes.

Discussion

It could be shown that there was a spontaneous transfer of phosphatidylcholine into sheep red cell membranes from phosphatidylcholine liposomes as well as from human serum lipoproteins. The transferred phosphatidylcholine molecules contained mostly polyunsaturated fatty acids. The reason for this preferential transfer is unknown so far.

Beside the protein-mediated lipid transfer [16], there are two possible mechanisms of lipid exchange between membranes: (1) collision complex model, first outlined by Gurd [17], which suggests that lipid exchange between membranes takes place upon contact of the membranes as a result of collision. (2) Aqueous diffusion model, originally suggested by Hagerman and Gould [18] for the exchange of cholesterol, involves the partitioning of the lipid molecules out of the membranes into the aqueous phase, which then act as an intermediate in the equilibration process. Recent studies on the kinetics of transfer of a fluorescent phosphatidylcholine [19] and cholesterol and phosphatidylcholine [10,21] support the aqueous diffusion model. The rate-limiting step for lipid exchange seems to be the transfer of lipid from the donor bilayer into the aqueous phase. This desorption process involves a disruption of lipid-lipid

interactions in the bilayer and the formation of an accomodating surrounding in the aqueous phase for the diffusing lipid molecule. As soon as the diffusion molecule collides with another vesicle it is rapidly taken up into the bilayer.

It is supposed that phosphatidylcholine molecules with two unsaturated fatty acids are more mobile vertically to the bilayer than those containing a saturated fatty acid. It is therefore possible that they are more easily desorbed from a lipid bilayer than the more rigid phosphatidylcholine molecules with saturated fatty acids. Besides, it may well be that the former are more soluble in an aqueous phase (e.g. showing higher critical micelle concentration values) than the latter. A detailed study on the desorption rates for phosphatidylcholine molecules with different acyl chain lengths and degree of saturation could answer this question.

Another reason for the preferential transfer of PC molecules with highly unsaturated fatty acids could be a protein-lipid interaction in the acceptor membrane. It is possible that certain membrane proteins have a high affinity for unsaturated fatty acids and they preferentially bind and retain those phosphatidylcholine molecules along the exchange process. At present, it cannot be decided which of these hypotheses is true for our system. However, attention should be paid in lipid exchange studies to whether labelled lipids, e.g., from microorganisms, with unknown fatty acid composition are used.

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