Biochimica et Biophysica Acta 812 (1985) 321–332
Elsevier

BBA 72359

Acyl selectivity in the transfer of molecular species of phosphatidylcholines from human erythrocytes

P. Child [a,*], J.J. Myher [b], F.A. Kuypers [a], J.A.F. Op den Kamp [a], A. Kuksis [b] and L.L.M. Van Deenen [a]

[a] *Laboratory of Biochemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht (The Netherlands) and* [b] *Banting and Best Department of Medical Research, C H Best Institute, 112 College Street, Toronto (Canada)*

(Received June 12th, 1984)

Key words Phosphatidylcholine, Lipid transfer; Transfer protein, Acyl selectivity, (Erythrocyte)

This report describes the molecular species composition of phosphatidylcholines (PC) transferred from human erythrocytes to acceptor vesicles composed of cholesterol and single PC species in the presence of PC-specific transfer protein from bovine liver. The compositions of the PC isolated from the vesicles were determined by capillary GLC as the diacylglycerol trimethylsilyl ethers. The cellular PC species appearing in the acceptor vesicles were enriched in unsaturated species and showed a low content of dipalmitoyl PC compared to untreated erythrocytes. This trend was independent of the composition of the PC used to construct the acceptor vesicles and it was possible to determine that the relative rates of efflux of the palmitoyl-containing phosphatidylcholines decreased in the order: palmitoyl-linoleoyl > palmitoyl-oleoyl > dipalmitoyl and in the stearoyl series, stearoyl-linoleoyl > stearoyl-oleoyl. No clear trend was distinguished for the influence of chain-length on the efflux, thus preventing an unambiguous assignment of the order of removal of all species from the cell membrane. Results derived for arachidonoyl-containing species were compromised by evidence for oxidation occurring during incubations at 37°C. To confirm that acyl selectivity was also possible during transfer in the absence of the transfer protein, the efflux of ^{14}C-labeled soya PC and [^{14}C]dipalmitoyl PC from prelabeled erythrocytes was measured using plasma as the acceptor. As predicted by the chromatographic analyses, ^{14}C-labeled soya PC effused up to 10-times faster than [^{14}C]dipalmitoyl PC from the red cell membrane. Thus, the more rapid transfer of unsaturated PC cannot be explained entirely as a specificity of the transfer protein and is consistent with the hypothesis that intermolecular interactions involving PC molecules within the erythrocyte membrane, become weaker with increasing unsaturation. The results suggest a potential role of PC-specific transfer protein as a probe of the nature of PC interactions within biological membranes.

* To whom correspondence should be addressed at (present address): Ludwig Institute for Cancer Research, 9 Earl Street, Toronto, Ontario, M4Y 1M4, Canada.

Abbreviations. PC-transfer protein, phosphatidylcholine-specific transfer protein, TLC, thin-layer chromatography; GLC, gas-liquid chromatography, PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine, DPPC, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine, PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine, POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, DLPC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine

Introduction

The PC-specific transfer protein from beef liver is known to catalyze the exchange of PCs with a wide variety of fatty acyl structures, including parinaric acid [1], spin-probe labeled fatty acids [2,3] and those containing photolabels [4,5]. The ability of the protein to bind these widely different structures suggests that, while there is an absolute requirement for the phosphatidylcholine headgroup structure [6], the PC-binding site possesses little or no specificity for the fatty acyl component of the molecule

Under some circumstances, however, an apparent selectivity for specific fatty acyl species is expressed as, for example, in the experiments of Kamp and co-workers [6] which demonstrated that [^{14}C]dipalmitoyl PC is transferred to an acceptor membrane much more slowly than ^{14}C-labeled egg PC from donor liposomes composed of either DPPC or egg PC. Similar findings have been reported by Kuypers et al. [7], using the transfer protein from bovine liver, and by Schulze and co-workers [8] using a PC-transfer protein-containing supernatant fraction from rat liver. The basis for the fatty acyl effect in these experiments has been suggested to be a result of differences in the physical properties of the lipids and their packing at the membrane interface rather than specificity expressed by the binding site of PC-transfer protein itself [6,8,9].

According to this hypothesis, the exchange of PC from a complex biological membrane should also be accompanied by an acyl selectivity that reflects the physical properties of the various molecular species and their packing within the bilayer. The membrane of choice for a study of this type was clearly the human erythrocyte in view of the extent of knowledge concerning membrane changes induced by treatment with PC-transfer protein and the absence of internal membranes.

We have therefore undertaken to examine the composition of the PC molecular species removed from human erythrocytes during the exchange catalyzed by PC-transfer protein in the presence of vesicles composed of single, pure PC species. To monitor their efflux, we have analyzed the composition of the diacylglycerols derived from PC of the postincubation vesicles, by capillary GLC. The results demonstrate that the erythrocyte PCs appearing in the vesicles are enriched in unsaturated species, regardless of the level of unsaturation in the original vesicles. This observation was confirmed independently by the demonstration of a more rapid efflux of ^{14}C-labeled soya PC, compared to [^{14}C]dipalmitoyl PC, from prelabeled cells. The relevance of these findings to an elucidation of the nature of intermolecular interactions within a biological membrane bilayer is discussed.

Materials and Methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were generously provided by W. Geurts van Kessel (Biochemistry Department, University of Utrecht, Utrecht, The Netherlands). Egg phosphatidylcholine, egg phosphatidic acid and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC) were purchased in the highest purity available from Sigma (St. Louis, MO, U.S.A.) and were used without further purification. Tri[9,10-^{3}H]oleoylglycerol, 1,2-di[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine were purchased from Amersham International (Amersham, Bucks., U.K.). Soya phosphatidyl[*N-methyl*-^{14}C]choline was available in the laboratory from previous studies [7]. Species analysis of unlabeled soya PC showed that it was composed of 28% PLPC, 38% DLPC, and contained no fully saturated species. The radioactive triacylglycerol and phosphatidylcholines gave one band following thin-layer chromatography on silica-gel H plates developed with ether/hexane (85 : 15, v/v) and chloroform/methanol/acetic acid/water (75 : 4. 12 : 6, v/v) [10], respectively. Scans of the resulting TLC plates showed that more than 95% of the radioactivity migrated with the carrier compound indicated by exposure to iodine.

Phosphatidylcholine-specific transfer protein from bovine liver was purified and prepared for experimental use as previously described [7,11].

Fresh human erythrocytes were obtained by venapuncture using acid/citrate/dextrose as anticoagulant. The cells were centrifuged for 10 min at

900 × *g* and the plasma and buffy coat carefully removed. The pellet was dispersed in 5 vol. buffer comprising of 150 mM NaCl/25 mM glucose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) (referred to as 'buffer' throughout) and recentrifuged. This washing procedure was repeated three times. The erythrocytes were used within 2 h of their preparation.

Preparation of vesicles. The vesicles in each case contained 47 mol% PC, 47 mol% cholesterol, 6 mol% egg phosphatidic acid and trace amounts of [^{14}C]PC and tri[^{3}H]acylglycerol giving a final specific radioactivity of $1.5 \cdot 10^5$ ^{14}C dpm/nmol PC with $1.5 \cdot 10^6$ ^{3}H dpm/nmol PC. The various components were dispensed from stock solutions in organic solvent into a rotary flask and dried by rotary evaporation. For vesicles prepared from DPPC, DOPC or POPC, the dried mixtures were dispersed in buffer (37°C) to give a final concentrations of 4.9 μmol PC/ml. The dispersions were then sonicated under nitrogen with a Branson Sonifier fitted with a standard probe (10 min at 0°C, 50 W) and subsequently centrifuged at 100000 × *g* for 30 min. The pellet was discarded and the supernatant used in the experiments described.

A modified procedure was used for the preparation of DLPC vesicles because of the risk of peroxidation of this species during sonication. In this case, the dried lipid mixture was dissolved in 100–200 μl ether and released, by syringe, into buffer at 65°C under nitrogen. The suspension was then centrifuged as above. The yield of vesicles after this procedure was typically 10–20% compared to more than 50% with the sonication method.

Incubation conditions. All incubations with the transfer protein were carried out essentially as described by Kuypers et al. [7]. They were performed in duplicate in plastic liquid-scintillation vials at 37°C in a shaking water-bath and typically contained washed erythrocytes (1 ml, 1120 nmol PC), vesicles (1230 nmol PC) and PC-transfer protein (240 μg protein) in a final volume of 3.6 ml buffer. After 3 h, the mixtures were centrifuged 10 min at 900 × *g* and the supernatants (donor vesicles) were removed. This fraction was further centrifuged for 5 min at 10000 × *g* to remove erythrocytes and large membrane fragments. Erythrocytes isolated from the first centrifugation were washed a further three times with 5 vol. buffer. In some instances, a fraction of the washed erythrocytes was reincubated with fresh vesicles and PC-transfer protein for a further 3 h.

Isolation of PC for determination of species composition and radioactivity. Total lipid extracts of erythrocytes were prepared according to the method of Rose and Oklander [12] and following evaporation were taken up in 6 ml chloroform/methanol (2 : 1. v/v). This organic solution was washed with 1.5 ml 0.9% saline and after removing the aqueous phase, evaporated and taken up in a small volume of chloroform/methanol for TLC.

Incubation supernatants (donor vesicles in approx. 2.5 ml vol.) were dispersed in 3.3 ml methanol and partitioned with 6.6 ml chloroform according to the method of Folch et al. [13]. The organic phase was then evaporated and redisolved in a small volume of chloroform/methanol (2 : 1, v/v) prior to TLC.

The extracts were chromatographed on silica-gel-H TLC plates developed with chloroform/methanol/acetic acid/water (75 : 45 : 12 : 6, v/v) [10] and the phosphatidylcholines isolated as described by Myher and Kuksis [14]. Samples of the resulting PC extracts were taken for liquid-scintillation counting and phosphorus determination. The analyses were carried out as described earlier [15] and the extent of PC-transfer protein-mediated PC replacement calculated according to Kuypers et al. [7].

The remainder of the PC extracts were treated with phospholipase C (*Clostridium welchii*, 1 I.U./mg PC) [16] and the resulting 1,2-diacyl-*sn*-glycerols derivatized with a mixture of pyridine/hexamethyldisilazane/ trimethylchlorosilane for GLC [16]. All extraction and derivatization procedures were carried out under nitrogen and in the presence of butylated hydroxytoluene to inhibit oxidation.

Gas-liquid chromatography. Capillary GLC of the tms ethers of the diacylglycerols was carried out isothermally at 250°C on a Hewlett-Packard Model 5880 automatic gas chromatograph equipped with a 10 m open tubular glass column (0.25 mm internal diameter) wall-coated with SP-2330 liquid phase (Supelco, Bellefonte, PA, U.S.A.) as reported by Myher and Kuksis [14]. Samples

from experiments using DLPC were chromatographed on a Packard series 730 gas chromatograph employing glass tubular columns (2 m × 2 mm internal diameter) packed with 3% Silar 5CP on Gas Chrom QII (80/100 mesh – Supelco, Bellefonte). Samples were injected directly onto the column packing and eluted isothermally at 270°C with a nitrogen flow-rate of 30 ml/min [16]. Major species were identified by comparison with the retention times of authentic standards and the remainder by comparison with reported retention times [14,16]. The peak areas used in the subsequent calculation were uncorrected.

The efflux of [^{14}C]PC from pre-labeled erythrocytes. The rate of efflux of [^{14}C]DPPC and ^{14}C-labeled soya PC from prelabeled cells was measured using a modification of the technique described by Lange et al. [17] for the analysis of cholesterol exchange. The cells were first labeled using donor vesicles containing cholesterol, egg phosphatidic acid and either DPPC with a trace amount of di[1-^{14}C]palmitoyl PC or DLPC with a ^{14}C-labeled soya PC tracer in a 3 h incubation with PC-transfer protein. After the incubation, the cells were centrifuged and washed well with buffer to remove adherent labeled vesicles. Incubation solutions were prepared in triplicate, containing the prelabeled cells (100 μl, 0.15 μmol PC), heat-inactivated plasma (450 μl, 1.0 μmol PC) and buffer to give a final hematocrit of 10%. Samples (100 μl) were withdrawn from each solution at various times, dispersed in 0.5 ml buffer and centrifuged (0.5 min, 8000 × *g*). The supernatant was removed and placed directly in a liquid-scintillation vial followed by 4.5 ml Instagel liquid-scintillation emulsifier cocktail (Packard Becker B.V., Groningen, The Netherlands). The cell pellet (typically 10 μl) was washed twice with 0.5 ml buffer and lysed with 20 μl distilled water. An extract of the cell lysate was made with 0.5 ml isopropanol and following extensive vortexing and centrifugation, the extract was transferred to a scintillation vial followed by 4.5 ml Instagel. The recovery of the labeled PC from the cells by this technique was not significantly different from that of control cells extracted with chloroform/methanol using standard procedures [13]. The incubation supernatant and the erythrocyte extracts (in triplicate) were analysed in a Prias PLD liquid-scintillation counter (Packard). The data was expressed as the ratio of the dpm present in the plasma against the total dpm in the sample at each time-point.

Results

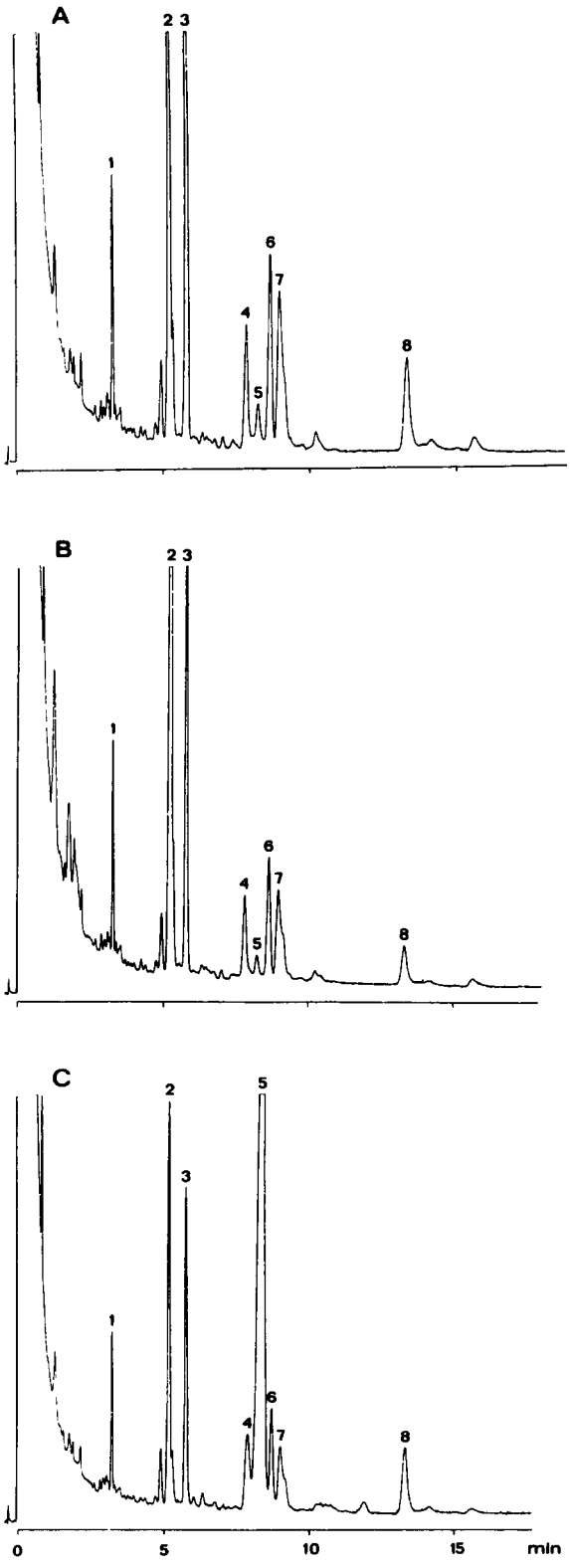
Purity of PCs used in donor vesicles

The method of analysis in these experiments relies chiefly on the appearance of erythrocyte PC species in vesicles initially composed of single species; therefore, it was important to establish the degree of purity of the acceptor PCs. Analysis of diacylglycerols liberated from the synthetic PCs by phospholipase C indicated the following: the DLPC preparation contained no other detectable compounds, the DPPC preparation contained less than 1% PSPC, the POPC sample contained 1% DOPC and 1% oleoyl-linoleoyl PC, while the DOPC preparation contained 3% SOPC, 4% POPC and less than 1% DPPC. In cases where the quantitation of erythrocyte PC species appearing in the vesicles would be influenced by these contaminants, the data was not used in subsequent calculations except for the case of DPPC in the DOPC preparation. In this instance, a correction was made for the amount of DPPC present in the original vesicle PC material.

Determination of PC exchange on a mass basis by GLC

The analysis of molecular species of PC by both capillary GLC and packed column GLC permits the resolution of all of the major compounds present in the human erythrocyte. A profile obtained from untreated erythrocytes is shown in panel A of Fig 1. POPC and PLPC account for more than 50% of the total peak area. SOPC and SLPC account for a further 15% of the total. In both the palmitoyl and the stearoyl series, the ratio of linoleoyl- to oleoyl-containing molecules is about 1.25 : 1. Dipalmitoyl PC and the arachidonoyl-containing PC species account for about 7 and 20% of the total, respectively. These area percentages are in general agreement with those based on argentation-TLC published by Marai and Kuksis [18] and Van Golde et al. [19].

The enrichment of the content of the donor species in the cell membrane that accompanies incubation of erythrocytes with PC-transfer pro-



tein and donor vesicles is clearly evident in panels B and C of Fig. 1. These illustrate the PC profiles of cells incubated with POPC- and DOPC-containing vesicles, respectively, to a level of about 25–40% replacement. In both cases, the extent of replacement was calculated from the change in the area percentage of the peak corresponding to the donor species – peak 2 in panel B and peak 5 in panel C. Taking into account the amount of native POPC and DOPC that effused during these changes, the calculated percent replacement based on the chromatographic data agreed well with those determined by radioactivity. As with determinations based on the cellular uptake of radioactive PC molecules however, the chromatographic method overestimated the replacement unless corrected for the presence of adherent vesicle material.

To investigate the practicality of determining cellular PC species appearing in the vesicle population, erythrocytes were incubated with vesicles composed of DPPC and cholesterol in the presence of PC-transfer protein. Control incubations were carried out in the absence of PC-transfer protein. Profiles of the PC species extracted from the supernatants following the exchange are presented in Fig. 2. It is evident from panel A that in the absence of PC-transfer protein, negligible amounts of erythrocytes PC species appeared in the donor vesicles under these conditions. From these data, the extent of spontaneous exchange was calculated to involve less than 2% of the cellular PC in 3 h.

With the addition of PC-transfer protein to the system, the appearance of DPPC in the cells is accompanied by the efflux of native PC species to the incubation medium. This is strikingly evident in panel B of Fig. 2. The concurrent increase in the

Fig. 1 Gas-liquid chromatographic profiles of diacylglycerol trimethylsilyl ethers derived from PC of modified erythrocytes Diacylglycerol trimethylsilyl ethers were prepared from erythrocyte PC as described in Materials and Methods. The compounds were chromatographed on a 10 m SP-2330 column isothermally at 250°C. The profiles represent material derived from untreated erythrocytes (A), erythrocytes treated for 3 h at 37°C with PC-transfer protein in the presence of vesicles composed of cholesterol and POPC (B), and erythrocytes treated with PC-transfer protein and vesicles of cholesterol and DOPC (C). Peak identification is as follows: 1, DPPC, 2, POPC, 3, PLPC, 4, SOPC, 5, DOPC, 6, SLPC, 7, PAPC; 8, SAPC

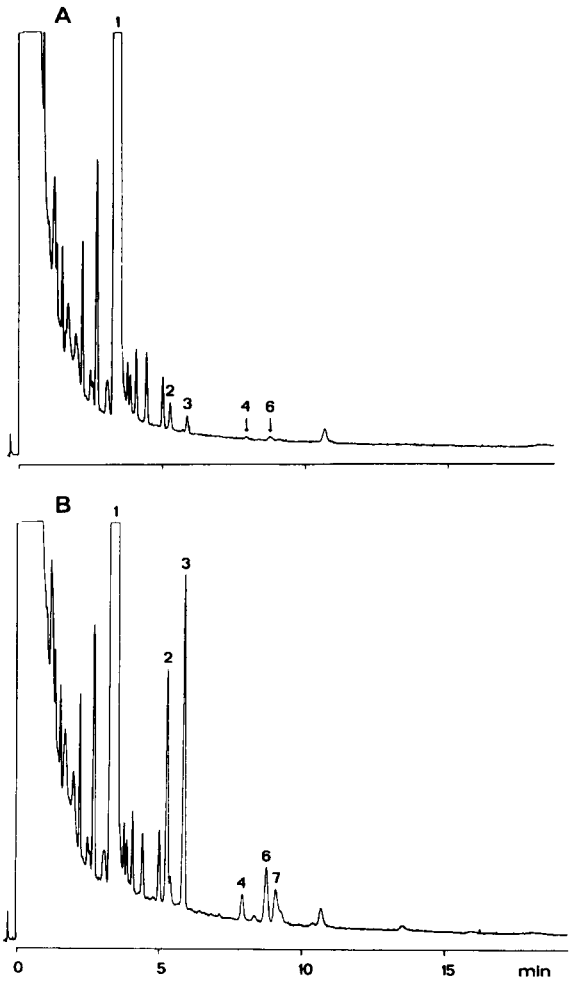


Fig. 2 GLC Profiles of diacylglycerol trimethylsilyl ethers derived from vesicle PC following incubations with erythrocytes in the presence and absence of transfer protein The vesicles were initially composed of pure DPPC/cholesterol/egg phosphatidic acid (0.47 : 0.47 : 0.06, mol%), and were incubated with erythrocytes at 37°C for 3 h with (B) or without (A) transfer protein Diacylglycerol trimethylsilyl ethers were prepared for vesicle PC and chromatographed as described in Materials and Methods Peak identification is as in Fig. 1

cellular DPPC content (not shown) cannot, therefore, be entirely the result of nonspecific adsorption of vesicles to the cell surface. Similar results were found with the other vesicle types, confirming, on a mass basis, the occurrence of an exchange process.

Calculation of the extent of replacement based on the amounts of native PC appearing in the incubation medium agreed closely with that calculated from the radioactivity appearing in the cell The replacement based on the vesicle species analysis was calculated to be 14% (sum of the area % of erythrocyte PC species multiplied by the ratio of vesicle to cellular PC) while the radioactive analysis, after correcting for adherent vesicles, gave 15%.

The selectivity of the exchange process

The first evidence of selectivity expressed during the removal of PC from the erythrocyte membrane is apparent when the composition of cellular PC species is plotted against time, as shown in Fig. 3 for POPC-treated cells. The uptake of POPC is accompanied by the efflux of native species and it is evident that the rates of transfer are not identical. Particularly, the content of DPPC does not decrease as rapidly as other species during the incubation. This suggested a slower rate of efflux for this molecule compared to the others, but other influences such as oxidation of the more un-

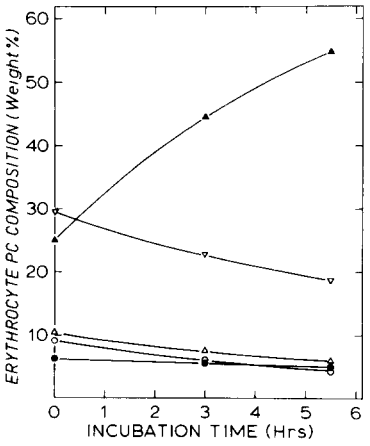


Fig. 3 Change in the erythrocyte PC species composition during incubation with PC-transfer protein and cholesterol/POPC vesicles. Cells were incubated in the presence of transfer protein and vesicles composed of cholesterol/POPC/egg phosphatidic acid (0.47 0.47 . 0.06, mol%) for the times indicated Erythrocyte PC was isolated and prepared for capillary GLC as described in Materials and Methods. The weight percent of each PC species was determined as the peak area for each divided by the total The species represented in the graph are POPC (▲); PLPC (▽); SOPC (△); PAPC (○) and DPPC (●) The results are the mean of values derived from duplicate reaction mixtures

saturated PCs had to be ruled out before drawing firm conclusions. The more sensitive indicator of selectivity – the appearance of particular erythrocyte PC species in the vesicle population – was therefore brought into use.

Comparison of the cellular PC appearing in the vesicles (e.g., panel B, Fig. 2) with those of untreated cells (panel A, Fig. 1) shows that all native species are capable of exchange. The chromatographic profile of the molecules removed is qualitatively similar to that of the original membrane. There are quantitative differences, however. In Table I, which summarizes the percent composition of species in the postincubation vesicles and in preincubation cells, the erythrocyte species transferred to DPPC vesicles are clearly enriched in PLPC. This is also the case in the postincubation POPC vesicles but the effect is much less obvious. In either case, the incubation medium is relatively enriched in linoleoyl-containing species derived from the cell. The indication of a slow rate of efflux of cellular dipalmitoyl PC in Fig. 3 is confirmed by these data.

To compare the extent of the selectivity measured with the various donor vesicles, ratios of different species in the postincubation supernatants, in postincubation cells and in untreated cells were calculated based on the area percentages obtained from the chromatograms. These are presented in Table II. It should be pointed out that for an increase in a ratio measured from species appearing in the vesicles to be considered reliable, it must be accompanied by a decrease in the corresponding ratio measured in the postincubation cells. Most of the comparisons presented in this table are in this way self-consistent. In cases where this relationship is not obeyed, for example in the comparison of the ratio PAPC/SLPC, other factors such as oxidation or inadequate integration of the peak areas were considered as influences.

To test the influence of unsaturation, the ratios of several pairs of species having identical fatty acids at the *sn*-1-position and differences in unsaturation at the *sn*-2-position were compared. With all of the vesicle compositions tested, the ratio PLPC/DPPC is much greater in the postincubation medium than in the original cells, indicating a more rapid efflux of PLPC than DPPC. Similarly, the ratios of PLPC/POPC and SLPC/SOPC were greater in the postincubation medium in all series tested. There is some evidence to suggest that the trend continues to include PAPC in the POPC-treated cells, that is, the ratio of PAPC/SLPC is greater in the vesicles than in the cells after 3 h of incubation (25% replacement) but there is clearly evidence for oxidation at 5 h (40% replacement). There is furthermore, no evidence of similar selectivity in the DPPC-treated cells. In view of this and the difficulty in accurately integrating the peak area of PAPC, these results were not interpreted further.

To test the influence of chain-length, several

TABLE I

PC SPECIES MIGRATION FROM ERYTHROCYTES INTO VESICLES

Human erythrocytes were incubated for 3 h with transfer protein and vesicles composed of either DPPC/cholesterol (left-hand columns) or POPC/cholesterol (right-hand columns). During the incubation, replacement of the cellular PC occurred to the extent of 15% with DPPC/cholesterol vesicles and 25% with POPC/cholesterol vesicles. Weight percentages were calculated from chromatographic peak areas, omitting the species used to construct the donor vesicles The data are the means of values derived from duplicate reaction mixtures.

PC species (wt%)	Initial vesicle PC: DPPC		POPC	
	preincubation erythrocytes	postincubation vesicles	preincubation erythrocytes	postincubation vesicles
DPPC	–	–	8	3
POPC	31	31	–	–
PLPC	39	44	39	41
SOPC	5	4	9	10
SLPC	11	11	14	19
PAPC	9	6	12	17

TABLE II

SUMMARY OF PC SPECIES RATIOS OCCURRING IN POSTINCUBATION VESICLES, POSTINCUBATION ERYTHROCYTES AND UNTREATED ERYTHROCYTES

The ratios were calculated from chromatographic peak areas following analyses of the PC derived from untreated erythrocytes and those incubated with transfer protein and vesicles composed of cholesterol, egg phosphatidic acid and the single PC species indicated in the table Values are the means of duplicate analyses. Percent replacement of erythrocyte PC by species originating in the donor vesicles are given within parentheses. 12–25% replacement was typically achieved after a 3-h incubation, while 40% required up to 6 h at 37°C RBC, red blood cells

Test ratio	PC species in donor vesicles		Untreated RBC	Postincubation	
				Vesicles	RBC
PLPC/DPPC	POPC	(25)	4 73	15 9	4 10
	POPC	(40)	4.10	9 34	3 66
	DOPC	(45)	4.73	8 81	2 86
	DLPC	(12)	3.6	5.77	3 66
PLPC/POPC	DPPC	(15)	1 27	1 45	1 25
	DLPC	(12)	1 21	1 28	1 16
SLPC/SOPC	POPC	(25)	1 58	2 00	1.43
		(40)	1 43	1.81	1.46
	DPPC	(15)	2 08	2 84	2.06
PAPC/SLPC	POPC	(25)	0 87	0 89	0 79
		(40)	0.79	0 56	0 71
	DPPC	(15)	0 65	0 63	0 65
PLPC/SLPC	DPPC	(15)	3 41	3 85	3 31
	POPC	(25)	2 76	2 19	3 02
		(40)	3 02	1 98	3 21
POPC/SOPC	DPPC	(15)	5.57	7.53	5 44
	DLPC	(12)	2.55	1.50	3 62
PAPC/SAPC	POPC	(25)	1 43	2 61	1 66

species were compared having identical fatty acids at the *sn*-2-position and palmitate or stearate at the *sn*-1-position. In experiments using DPPC vesicles, cellular species having palmitate at the 1-position are clearly present in larger proportions in the medium than stearoyl species. In experiments using POPC- or DLPC-treated cells, however, the opposite was true. Thus, in contrast to the influence of unsaturation on the rate of efflux, there was no consistent trend concerning the effect of chain-length in these experiments.

Efflux of [^{14}C]DPPC and ^{14}C-labeled soya PC from erythrocytes

The results of the previous section demonstrate that the PC-transfer protein-mediated efflux of PC from human erythrocytes occurs with a preferential release of unsaturated species and with a less clear dependence on chain-length. It was therefore important to determine if a similar selectivity was manifest during exchange in the absence of transfer protein. For this purpose, plasma was chosen as the acceptor, as the exchange of PC between erythrocytes and plasma has been reported to occur at a rate of about 1% per h [20]. Because of the complexity of the plasma PC species, the analysis necessitated the use of radiolabels rather than GLC and for this purpose ^{14}C-labeled soya PC and [^{14}C]DPPC were chosen based on the prediction that widely different rates of efflux would occur between fully saturated and polyunsaturated PC molecules. The labeled PC species were incorporated to the extent of 10% replacement in the case of soya PC and 15% with DPPC in separate cell populations. Thus, the labeled pool in the soya PC-treated erythrocytes represented about 10% of

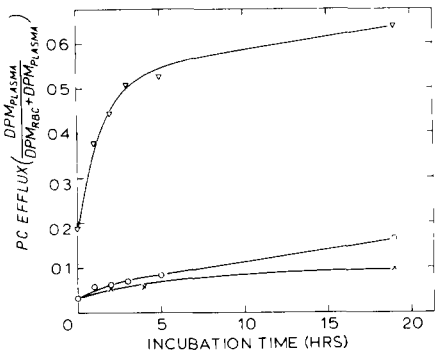


Fig 4 Efflux of radiolabeled soya PC and DPPC from prelabeled human erythrocytes Cells were prelabeled during a 3 h incubation with PC-transfer protein and vesicles composed of either soya PC (plus trace soya phosphatidyl[*N-methyl*-^{14}C]choline/cholesterol or DPPC (plus trace di[^{14}C]palmitoyl PC)/cholesterol. Efflux was subsequently measured during incubation of the cells with heat-inactivated human plasma Efflux was determined as outlined in Materials and Methods, and the data are expressed as the fraction of the total radioactivity that is present in the plasma (supernatant) at each time point The symbols represent soya PC (▽), DPPC (○) and red cell hemolysis (×) and are the means of values derived from triplicate reaction mixtures

the cellular PC, while the DPPC exchange in the other preparation increased the DPPC pool from 7 to 20% of the total cellular PC.

The efflux of the radioactive molecules with time is depicted in Fig. 4. In support of the data derived on a mass basis, the efflux of ^{14}C-labeled soya PC is markedly faster than that of [^{14}C]DPPC. Cell lysis occurring during the incubation was identical in both cell types and did not exceed 10% in 20 h of incubation. Within the first 3 h, the rate of efflux averaged about 1% per h for [^{14}C]DPPC and 10% per h for ^{14}C-labeled soya PC. Subsequently, the rates averaged 0.8% per h and 0.6% per h for soya PC and DPPC, respectively.

Discussion

Overview of methodology

Using the methodology described in this report, we have monitored PC-transfer protein-mediated exchange on a mass basis, looking specifically for evidence of a selectivity during the extraction of PC molecular species from the human erythrocyte membrane. The method represents a significant improvement over the analysis of fatty acids for this purpose, in that each chromatographic peak can be assigned, in most cases, to one unique molecular species of PC, avoiding the confusion that may arise from the occurrence of each fatty acid in more than one type of PC molecule.

A further helpful innovation was the use of vesicles composed of a single pure PC species to serve as acceptor for PC effusion from the erythrocytes during incubation with PC-transfer protein. The appearance of cellular species can in this way be quantitated against a low background and therefore with predictably greater accuracy than the determination of small changes in the peak areas of large GLC peaks as occurs, for example, in extracts from the cells themselves. To overcome the lack of information over the particular species used to construct the vesicles, different compositions were used, covering a wide range of unsaturations and chain-lengths.

The analyses were facilitated by a low level of spontaneous exchange – less than 2% of the total erythrocyte PC – occurring during incubations in the absence of transfer protein. The appearance of cellular PC in the vesicles on addition of PC-transfer protein could therefore be confidently assumed to arise through an exchange process rather than contamination of the supernatant by membrane fragments. The appearance of these species, in addition to providing information on the selectivity, also allowed a simple confirmation of the extent of replacement based on the area percentage of the cellular PC in extracts of the vesicles. This method required an estimate of the contribution of, for example, erythrocyte DPPC to the weight percentage of this peak in the chromatograms when DPPC vesicles were used, but for this purpose the exchange was considered to be nonselective. The estimates of the extent of replacement using this method were in excellent agreement with those calculated from the cellular uptake of radioactive tracers and corrected for adherent vesicles using tri[^{3}H]oleoylglycerol. No such correction is required with the chromatographic method and the ratio of the size of the PC pools was the only additional information required.

Selectivity of the exchange process mediated by the transfer protein

It is clear from the evidence obtained using

vesicles composed of DPPC and POPC, presented in Table I, that the PC transferred from erythrocytes is enriched in unsaturated species. In the experiments represented in this table and in fact with all vesicles compositions tested, the incubation medium became enriched in linoleate-containing species (compared to oleate-containing species having the same number of carbon atoms). This was associated with a corresponding decrease in the content of linoleoyl-containing PC in the cells themselves. These results reflect a faster rate of efflux of the linoleate species and rule out a selective oxidative destruction of the diunsaturated species as an explanation of the decreased cellular content.

Oxidation may, however, have influenced results involving arachidonate-containing PC. Data in Table II indicate that the ratio of PAPC/SLPC in the incubation medium was higher than that in the cells treated with POPC vesicles up to a level of 25% replacement, but at higher replacements the ratio in both postincubation cells and vesicles dropped to less than in the original erythrocytes. The results therefore, are not self-consistent and oxidation of PAPC must be considered as a factor in this inconsistency.

One of the slowest species to effuse from the membrane was DPPC. This is evident from the relatively slow decrease in the weight percentage of this molecular compared to the other native species in Fig. 3, and also in the small amount of DPPC appearing in the postincubation vesicles. This is particularly evident in data presented in Table I.

From the ratios in Table II (omitting the data for arachidonoyl-containing species), we conclude that the rates of efflux from linoleoyl- and oleoyl-containing molecules, and DPPC, decrease in the order: PLPC > POPC > DPPC, and SLPC > SOPC, with DPPC apparently the slowest to effuse from the erythrocyte membrane. It is not possible to conclude from this data that the structure of the vesicle PC has no influence on the rate of efflux of erythrocyte diacylphospholipids but it is clear that the general trends shown above are consistent through a series of vesicle compositions ranging from completely saturated PCs with 32 carbons to molecules possessing 36 carbons and 4 double bonds.

An interdigitation of the two series shown above would have to be based on clear data regarding the influence of chain-length on the rate of PC efflux. This parameter was not, however, independent of the structure of the vesicle PC composition. A comparison, for example, of the PLPC/SLPC ratio in Table I shows that it is greater in the vesicles than in the cells in the DPPC experiment but smaller when POPC was used to construct the vesicles. Thus, the lower molecular weight species effused to a greater extent in the DPPC experiments, but the opposite was true when the vesicles were composed of POPC or DLPC. It therefore appears likely that the structure of the incoming PC plays a role in determining the selectivity of efflux of molecules of differing chain-lengths. Initial rate data, in addition to the equilibrium results derived from the current experiments, will be required to further refine this concept.

PC-transfer protein as a probe of membrane organization

The use of PC-transfer protein as a probe of the characteristics of the interface formed by PC within a membrane bilayer, as suggested in this report, is predicated on the absence of acyl specificity in the binding site of the protein itself. Initially, this thesis was supported by three pieces of information: Firstly, as described in Introduction, the protein is able to transfer PCs containing a wide variety of bulky fatty acyl derivatives. Secondly, Bozzato and Tinker [9] have demonstrated that the exchange of DPPC mediated by PC-transfer protein is markedly dependent on the structural properties of the water/bilayer interface, indicating that previous rate differences with different PC species may be related to their interfacial properties. Thirdly, it has been demonstrated using transfer protein from rat liver that there is no detectable preferential transfer of any molecular species between microsomes and mitochondria when the PC subclasses are partially resolved by AgNO3-TLC [21,22], and yet, as with the protein from beef liver, preferential transfer of unsaturated PC was found when vesicles made of pure PC were used [8].

All of these findings suggest that acyl selectivity in the protein-mediated PC transfer reflects differences in the nature of the interface formed by, or surrounding, different species of PC. To test

that membrane properties were indeed an underlying factor in the results of our experiments, we chose to examine the efflux of PC from prelabeled cells into plasma. For this purpose, we chose [^{14}C]DPPC and ^{14}C-labeled soya PC, the rates of efflux of which, we predicted, should be widely different.

The results presented in Fig. 4 demonstrate that the efflux of the unsaturated mixture of species is indeed much more rapid than that of DPPC. It should be mentioned that these rates were not measured concurrently from the same cell but from two populations previously treated with vesicles of the appropriate radioactive PC and PC-transfer protein to build in the radiolabel. With these differences in pretreatment and subsequent erythrocyte PC species composition, the comparison cannot be expected to be as reliable as that based on the GLC method, but the dramatic differences in the rates of efflux obviously cannot be attributed entirely to this factor. These observations are similarly not expected to be related to any preference for one or the other species expressed by the acceptor lipoproteins, in view of the reported absence of any selectivity during the exchange of PC between ^{32}P-labeled rat plasma and rat erythrocytes, when monitored by fractionation of the PC species by argentation TLC [20].

These findings support the interesting possibility that the exchange protein response to differences in the strength of intermolecular interactions within the PC pool of biological membranes. This leads immediately to the conclusion that interactions occurring between unsaturated molecules are weaker than those between saturated ones in the erythrocyte membrane. A similar dependency of intermolecular interaction on structure has been described in reports of model experiments that include demonstrations of a greater acyl chain mobility in vesicles composed of pure unsaturated PC species compared to the mobility of saturated phosphatidylcholines [23], and the finding that the miscibility of various phosphatidylcholines with fully saturated species is reduced by the introduction of double bonds [24]. The structure-dependent interaction of PC and diacylglycerols with saturated hydrocarbons is also used analytically as the basis for the resolution of molecular species of these glycerolipids by reversed-phase HPLC [25–27]. The basis for the more rapid elution of unsaturated species in the latter case has been suggested to be a smaller available contact area for hydrophobic interaction between unsaturated acyl chains and the saturated hydrocarbon stationary phase than between saturated molecules and the stationary phase [25]. It appears from our results that qualitatively similar interactions may occur within the red cell membrane despite the complexity of the lipid milieu and the presence of a wide variety of nonlipid molecules. Previous reports of a more rapid transbilayer 'flip-flop' of unsaturated PC series, compared to saturated, in the erythrocyte membrane [20,28] support this conclusion. The extremely slow efflux of DPPC may therefore be a reflection of an extremely strong Van der Waal's interaction between this species and others in the membrane based on the high contact area provided by the two fully saturated chains. This factor may be responsible for the occurrence in vivo of a significantly higher mole percentage of fully saturated PC species (DPPC and PSPC) in erythrocytes than in the surrounding plasma in humans, rabbits and rats [18,19].

This study demonstrates the application of PC-transfer protein as a membrane probe in an examination of the acyl selectivity of PC species removed from the human erythrocyte membrane. This protein, in combination with capillary GLC, provides a convenient method for monitoring the transfer of all cellular PC species simultaneously and for this purpose represents a significant improvement over methods based on radioactivity and fatty acid analysis. Experiments are currently in progress, applying these useful tools to probe the influence of the structure of the bulk PC composition on the selectivity and rates of efflux of PC from human erythrocytes. In this manner, we hope to provide additional insight into the nature of the intermolecular interactions occurring in biological membranes.

Acknowledgements

The present work was carried out under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from The Netherlands Organization for the Ad-

vancement of Pure Research (Z.W.O.). P.C. is the recipient of a fellowship from the Medical Research Council of Canada.

References

1 Somerharju, P., Brockerhoff, H. and Wirtz, K W A (1981) Biochim. Biophys Acta 649, 521–528
2 Devaux, P F., Moonen, P., Bienvenue, A and Wirtz, K W.A (1977) Proc Natl Acad. Sci USA 74, 1807–1810
3 Machida, K and Ohnishi, S (1978) Biochim Biophys Acta 507, 156–164
4 Moonen, P., Haagsman, H.P., Van Deenen, L.L.M. and Wirtz, K.W.A. (1979) Eur. J. Biochem 99, 439–445
5 Wirtz, K W A., Moonen, P., Van Deenen, L L M, Radhakrishnan, R. and Khorana, H G (1980) Ann N.Y. Acad Sci 348, 244–255
6 Kamp, H H., Wirtz, K W A, Baer, P R, Slotboom, A J, Rosenthal, A F., Paltauf, F. and Van Deenen, L L M (1977) Biochemistry 16, 1310–1316
7 Kuypers, F A, Roelofsen, B, Op den Kamp, J A.F and Van Deenen, L L.M (1984) Biochim Biophys Acta 769, 337–367
8 Schulze, G., Jung, K, Kunze, D and Egger, E (1977) FEBS Lett 74, 220–224
9 Bozzato, R P and Tinker, D O. (1982) Can J. Biochem 60, 409–418
10 Skipski, V D, Peterson, R F. and Barclay, M (1964) Biochem J 90, 374–378
11 Kamp, H H and Wirtz, K.W A (1974) Methods Enzymol 32, 140–146
12 Rose, H G and Oklander, M. (1965) J Lipid Res 6, 428–431
13 Folch, J, Lees, M. and Sloane Stanley, G H (1957) J Biol Chem 266, 497–509
14 Myher, J J and Kuksis, A (1982) Can J Biochem 60, 638–650
15 Child, P and Kuksis, A (1980) Can. J Biochem 58, 1215–1222
16 Myher, J J. and Kuksis, A (1975) J Chromatogr. Sci 13, 138–145
17 Lange, Y, Molinaro, A L, Chauncey, T.R and Steck, T L (1983) J Biol Chem 258, 6920–6926
18 Marai, L and Kuksis, A. (1969) J. Lipid Res. 10, 141–152
19 Van Golde, L M G, Tomasi, V and Van Deenen, L L M (1967) Chem Phys Lipids 1, 282–293
20 Renooij, W and Van Golde, L M G (1977) Biochim Biophys. Acta 470, 465–474
21 Wirtz, K W A, Van Golde, L M G and Van Deenen, L L M (1970) Biochim. Biophys Acta 218, 176–179
22 Taniguchi, M, Hirayama, A and Sakagami, T. (1973) Biochim. Biophys Acta 296, 65–70
23 Lancee-Hermkens, A.M.W and De Kruijff, B (1977) Biochim Biophys Acta 470, 141–151
24 Lentz, B R, Barenholz, Y and Thompson, T (1976) Biochemistry 15, 4529–4537
25 Smith, M and Jungalwala, F.B (1981) J Lipid Res 22, 697–704
26 Pind, S, Kuksis, A, Myher, J J and Marai, L (1984) Can J Biochem. Cell Biol. 62, 301–309
27 Patton, G M, Fasulo, J M and Robins, S.J (1982) J Lipid Res 23, 190–196
28 Van Meer, G M. and Op den Kamp, J A F (1982) J Cell Biochem 19, 193–204