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Aminophospholipid molecular species asymmetry in the human erythrocyte plasma membrane

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The transbilayer distribution of the molecular species of aminophospholipids in human red blood cell plasma membrane has been investigated using a covalent labelling technique. Separation and quantitative analysis of the molecular species of phosphatidylethanolamine (PE) and phosphatidylserine (PS) was performed using high-performance liquid chromatography with UV detection of the trinitrophenyl derivatives obtained after reaction with trinitrobenzenesulfonic acid (TNBS). When the molecular species distribution obtained with intact cells was compared to that of the whole membrane, a molecular species asymmetry was evident. This phenomenon was most clearly evident when the reaction was performed at low temperatures (0 °C) and was obscured by the excessive labelling or probe permeation associated with higher temperatures or longer incubation times. The monoene species were enriched in the outer leaflet, they comprised about 30% of the PE species in this leaflet. The polyunsaturates were preferentially localized in the inner leaflet and this was true of the arachidonyl species in particular as they represented up to 35% of this pool. The *w*-3 polyunsaturated fatty acids displayed a preferential localization in the plasmalogen subclass in comparison to the diacyl fraction, i.e., they comprised about 58 of the former and 42% of the latter subclass of cellular PE *w*-3 species. Data concerning the separation, identification and quantification of PS molecular species in human erythrocytes is also presented. The internal localization of the polyunsaturated species as well as the compartmentalization of the *w*–3 and *w*–6 pools will have metabolic, structural and physical implications for membrane function.

Introduction

It is now established that the red blood cell (RBC) plasma membrane, as well as most mammalian cell membranes, possesses an asymmetric distribution of phospholipid classes between the two leaflets. The aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) as well as phosphatidylinositol (PI), are localized mainly in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin (SM) are found predominantly in the outer leaflet [1,2]. The fatty acyl content of these phospholipids may influence such membrane parameters as fluidity, permeability, enzyme or receptor activity and lipid-protein associations. [3,4].

In spite of the heterogeneity of the molecular species within lipid classes, there have been few reports of their transbilayer distribution. An asymmetrical species distribution has been reported for SM in the RBC plasma membrane [5]. In contrast, the 20 different molecular species of PC appeared to be randomly distributed between both leaflets of the RBC membrane due to a flip-flop process [6]. According to Marinetti and Crain [7], there appeared to be no significant difference in the fatty acyl composition of the outer or inner leaflet PE in the RBC membrane. However, a preliminary report showed that polyunsaturated PE species were con-

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Abbreviations: Alk-, alkenyl; BHT, 2,6-di-*tert*-butylcresol; BSA bovine serum albumin; DMA, dimethylacetal; EDTA, ethylenediamine tetraacetic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SM, sphingomyelin; TLE, total lipid extract; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl-.

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centrated on the membrane interior of human RBC but that monoenes were more likely to be on the cell surface [8]. In addition, an asymmetric distribution of PE molecular species has been found in other cell types [9–11]. Some studies have also indicated a non-random distribution of PE subclasses [4,12]. Therefore, we studied the topological transbilayer distribution of the aminophospholipids in the human RBC plasma membrane in greater detail using a recently developed HPLC method for species quantification [13]. We used the non-permeant reagent trinitrobenzenesulfonic acid (TNBS) as a covalent chemical probe; this probe has been successfully applied to the study of the aminophospholipid distribution in erythrocytes [14] and platelets [15] as well as bacterial [16] and many other cell types. A preliminary report of this work has been presented [17].

Materials and Methods

Red blood cell preparation

Blood was collected by venipuncture from human volunteers using acid citrate dextrose as anticoagulant [18]. After centrifugation at 250 × *g* for 20 min at room temperature, the platelet rich plasma (PRP), the buffy coat and a small amount of the top layer of RBC were removed. The cells were washed three times in cold isotonic buffer (Buffer A) containing 154 mM NaCl, 25 mM glucose and 10 mM Tris-HCl (pH 7.4) with centrifugation at 1000 × *g* for 15 min at 4°C. A small portion of the top of the erythrocyte layer was removed during each cycle in order to avoid leukocyte contamination. These packed RBC were immediately used for TNBS reaction and/or lipid extraction. Alternatively, they were stored in Buffer A containing 0.5% fatty acid-free bovine serum albumin (BSA) for 24 or 48 h for the purpose of a storage-stability study.

Reaction of intact erythrocytes with TNBS and separation of the cell surface trinitrophenyl-PE

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent was purchased from Pierce Chemical Co. (Rockford, IL) and used without further purification. Cell aminophospholipids were labeled with TNBS by a modification of the procedure of Marinetti and Love [14]. Unless otherwise stated, 1 ml of packed RBC were reacted with 15 ml of ice-cold freshly prepared TNBS solution (2 mM final concentration) containing 120 mM $NaHCO_3$, 40 mM NaCl and 4.4 mM glucose, (pH 8.3). The pH was adjusted at 0°C. They were incubated under non-penetrating conditions [16], i.e., for 30 min at 0°C using gentle agitation on an orbital shaker. Light-sheltered tubes were used in order to avoid photodecomposition. Excess reagent was removed by centrifuging at 1000 × *g* for 10 min at 4°C. The cells were then washed twice with ice-cold buffer A (pH 7.4); in the first wash, 0.5% fatty acid free-BSA was added to react with remaining TNBS reagent.

Lipids were extracted twice according to the procedure of Reed et al. [19]. The RBC were added dropwise into 2 ml of methanol containing 50 μg of 2,6-di-*tert*-butylcresol (BHT), 50 μl of 0.2 M EDTA and 10 nanomoles of di-14:0-trinitrophenyl-PE as internal standard. The cells were stirred for 5 min at 0°C and then 2 ml of chloroform were added and the extraction continued for another 5 min. After centrifugation, the supernatant was retained and the pellet reextracted. The pooled chloroform-methanol supernatants were separated into 2 phases by addition of water using the solvent ratios described by Bligh and Dyer [20].

The lower chloroform phase was concentrated with a stream of nitrogen, redissolved in 200 μl of chloroform/methanol (2:1, v/v), spotted on a silica gel plate (LK5, Whatman Inc., Clifton, NJ) and developed in a light-sheltered tank. TNP-PE derivatives, as visualized by their yellow color, were separated from unreacted PE and PS and from other phospholipid classes using the solvent system of chloroform/acetone/methanol (containing 100 μg BHT)/acetic acid/water (5:2:1:1:0.5, v/v) [21]. The plates were dried under nitrogen and the yellow bands were scraped and extracted twice with 3 ml of methanol containing 50 μg of BHT. The extracts were centrifuged at 3000 rpm at 4°C for 20 min in order to remove fine particles of silica gel. After evaporation under nitrogen, the extracts were dissolved in methanol. These TNP-PE derivatives corresponded to the PE of the outer leaflet of the plasma membrane. They were subfractionated into molecular species of TNP-PE and quantified by HPLC as described below.

The release of hemoglobin was estimated by centrifugation at 2500 rpm for 10 min after TNBS reaction and determination of the absorbance of the supernatant at 540 nm. Percentage hemolysis was obtained by comparison to the absorbance of non-treated cells which were lysed in water.

Preparation of erythrocyte total lipid extracts (TLE) and determination of plasma membrane aminophospholipids

Washed, packed RBC (1 ml) were extracted twice according to the procedure of Reed et al. [19] as previously described for the intact cells and in the presence of a mixture of di-14:0-PE and PS as internal standards. The pooled chloroform-methanol supernatants were then separated into 2 phases by addition of water according to Bligh and Dyer [20]. Aliquots of these chloroform extracts (RBC TLE) were removed to determine the total phospholipid phosphorus according to a modification of the method of Nelson [22]. The remaining samples were then derivatized with TNBS following a modification of the procedure described by Gordesky and Marinetti [23] for TNBS labeling of PE and PS in organic solution. Briefly, each RBC TLE

sample, containing about 4 μmoles of lipid phosphorus, was evaporated with a stream of nitrogen and dissolved in 1 ml of chloroform. Two ml of methanol containing 50 μg of BHT, 19 μl of 500 mM TNBS in methanol and 0.4 ml of 5% $NaHCO_3$ were added successively with vortex agitation. The light-sheltered tubes were then flushed with nitrogen and incubated at 30°C in a shaking water bath. After 30 min, another aliquot of $NaHCO_3$ and TNBS reagent (5 mM final concentration) was added and incubation continued for an additional 60 min. After cooling on ice, 1 ml of chloroform and 1 ml of 0.5 M HCl (to bring the pH to 5.0) were added so that two phases were formed [20]. The chloroform phase containing the yellow TNP-PE and TNP-PS derivatives was separated by thin-layer chromatography (TLC) as described above. After elution into methanol, aliquots were separated into molecular species and quantified by HPLC. These TNP-PE and TNP-PS derivatives corresponded to the total plasma membrane PE and PS composition.

Preparation of trinitrophenyl-aminophospholipid standards

Various molecular species of synthetic PE and PS were obtained from Avanti Polar Lipids (Birmingham, AL) and were derivatized with TNBS in organic solution and purified by TLC as described above. The molar extinction coefficients of the various molecular species of TNP-PE and TNP-PS at their absorbance maxima of 338 and 342 nm, respectively, in methanol were essentially the same, i.e., 17140 ± 750 [13]. These TNP molecular species standards were used to determine the retention behavior on the reversed phase column as well as the quantitative performance of the HPLC method. In addition, di-14:0-PE and PS or the di-14:0-TNP-PE derivatives were used as internal standards in the RBC extraction procedure, as appropriate.

Separation and quantification of molecular species of TNP-PE and TNP-PS by HPLC

Separation of molecular species of TNP-PE and TNP-PS was performed on an HP 1090 liquid chromatograph equipped with an HP 1040 diode array detector and an HP 79995A analytical workstation as previously described [13]. TNP-PE and PS were quantified by their absorbance at 338 and 342 nm, respectively. A linear relationship of peak area to molar amounts was found for the various TNP molecular species standards. They were separated on the RP-HPLC column according to differences in the chain length and degree of unsaturation in their fatty acid components. Since isocratic conditions were used during most of the analysis period for the TNP-PE species, a relationship between elution times and the fatty acid composition at the *sn*-1 and *sn*-2 positions was found for the various TNP-PE molecular species [13].

Fatty acid analysis

The identity of the various molecular species of TNP-PE and TNP-PS from the RBC membrane was determined by gas chromatographic (GC) analysis of each peak separated after HPLC analysis. Fractions collected after HPLC were separated into two phases using the solvent ratios described by Bligh and Dyer [20] and dried under nitrogen. Fatty acids were transmethylated with boron trifluoride in methanol (14% w/v, Sigma, St Louis, MO) according to Morrison and Smith [24] and extracted with hexane (Burdick and Jackson). The combined extracts were evaporated to dryness under nitrogen, redissolved in hexane and injected into an HP 5880 instrument equipped with a flame ionization detector. Helium was used as carrier gas with a linear velocity of 35 cm/s and nitrogen was the make-up gas. A 50 m × 0.25 mm id OV-351 capillary column with a 0.25 μm film-thickness (Analabs, Foxboro, MA) was used with an oven temperature program of from 200 to 225°C at 1 C°/min and held isothermal at 225°C thereafter. Fatty acid methyl ester and dimethylacetal (DMA) peaks were identified by their retention times based on a comparison to commercial standard mixtures (Nu-Check Prep, Elysian, MN).

Statistics

ANOVA analysis of variance was performed to compare the various species distributions (Fisher PLSD, Scheffe *F*-test, Dunnett *t*-test).

Results

Validation of the TNBS labeling procedure for membrane erythrocyte PE

The question of permeability of TNBS is critical in using this probe to locate the aminophospholipids on the outer surface of the membrane. Thus in preliminary experiments, we investigated the reaction conditions, i.e., time, concentration and temperature; conditions were subsequently chosen so that permeation of the reagent was avoided and a distribution of PE species representative of that on the cell surface was obtained. TNBS reacted quickly in the first 15 min and reached a plateau in the first two hours after which additional TNBS reaction was associated with the beginning of cell lysis. A similar finding was made regarding TNBS concentration as a plateau was reached in the 1–5 mM range and additional reaction at higher concentrations was associated with lysis. Also, TNBS permeation was associated with higher incubation temperatures as previously reported [25,26].

Under our standard non-penetrating reaction conditions (0°C, pH 8.3, 2mM TNBS, 30 min), the amounts

of RBC membrane PE labeled represented 0.81 ± 0.09% of the total cellular PE (mean ± S.E. of 18 determinations). Previous studies using chemical probes and/or phospholipase hydrolysis [1,25] have shown that 10 to 20% of the human RBC membrane PE was located in the outer leaflet. Thus our limited incubation conditions allowed us to label only 4 to 8% of the theoretical outer leaflet PE. This appears to be a necessary consequence of using conditions under which there is no TNBS permeation. However, the question may arise as to whether the PE species asymmetry noted herein is observed only with the limited labeling scheme prescribed by these conditions or is a more general characteristic of plasma membrane structure. This question was addressed by comparing the PE species distributions at various levels of TNBS labelling.

Fig. 1 shows a graphical representation of the proportions of the main PE molecular species in the outer leaflet as a function of time, TNBS concentration and temperature. A 'symmetry index' could be defined for each species from the ratio of its percentage in the outer leaflet divided by that in the total membrane. Thus, any ratio above 1.0 correspond to a species with a higher representation in the outer leaflet, whereas values below 1.0 correspond to those more highly represented in the inner leaflet. We did not observe any appreciable variation in the species distribution when the incubation was performed for either 15 or 120 min, when the TNBS concentration was raised from 1 to 5 mM, nor when the temperature was increased up to 15°C. However, further increases in incubation time, TNBS concentration and temperature led to a more symmetric distribution of the various PE species. This was concomitant with increased amounts of labeled PE as well as a higher degree of hemolysis indicating that the reagent penetrated the plasma membrane under these stronger conditions.

In order to show that the reaction of TNBS was not selective for any particular PE species, we compared the PE distribution after derivatization of the RBC TLE in organic solution at 30°C for 90 min to that from derivatization of cells lysed in hypotonic buffer in the presence of detergent (0.5% sodium deoxycholate) or after sonication. The PE species distribution showed little or no variation in these three cases, as expected. Also, the PE molecular species distribution of RBC lipid extracts sonicated in Buffer A and labelled with TNBS were the same as those labeled in organic solution (data not shown). These experiments indicate that all species of PE may react with TNBS in aqueous solution when they become accessible to the reagent. It therefore appears that despite the small proportion of PE labelled, these species were representative of the human erythrocyte plasma membrane surface pool. That only the cell surface was labelled was supported by our observations that no PS labeling could be detected

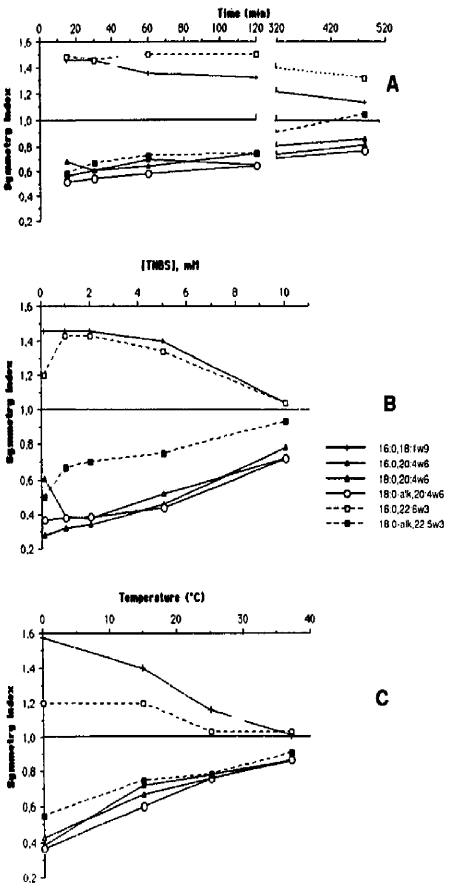

Fig. 1. Variations in the RBC PE species asymmetry as a function of time (A), TNBS concentration (B) and temperature (C). The major species were selected and their identifications are as indicated on the figure. Alk indicates an alkenyl linkage. The 'Symmetry Index' was calculated from the ratio of the mole% value of each species on the outer leaflet to that of the total membrane as described in Results.

when intact cells were reacted with TNBS and the percentage of cell lysis was very low (< 0.4%). These conditions were then used to explore in greater detail the phenomenon of PE molecular species asymmetry.

PE molecular species asymmetry in the human erythrocyte plasma membrane

When the PE fractions corresponding to the cell surface pool (or 'outer PE') and to the total lipid extract, assumed to be representative of the whole RBC plasma membrane, were analyzed by HPLC, a molecular species asymmetry was evident (Fig. 2). Molecular species were assigned after fatty acid and fatty aldehyde determination by GC analysis of each peak collected after HPLC separation. The identity of the alkenyl

species was further confirmed by their selective acidolysis (data not shown). The molar percentages of each species expressed for each PE subclass are presented in Table I. We observed that monoene species ($w-9$) were more represented on the cell surface, whereas polyenes ($w-6 + w-3$) were more concentrated in the total lipid

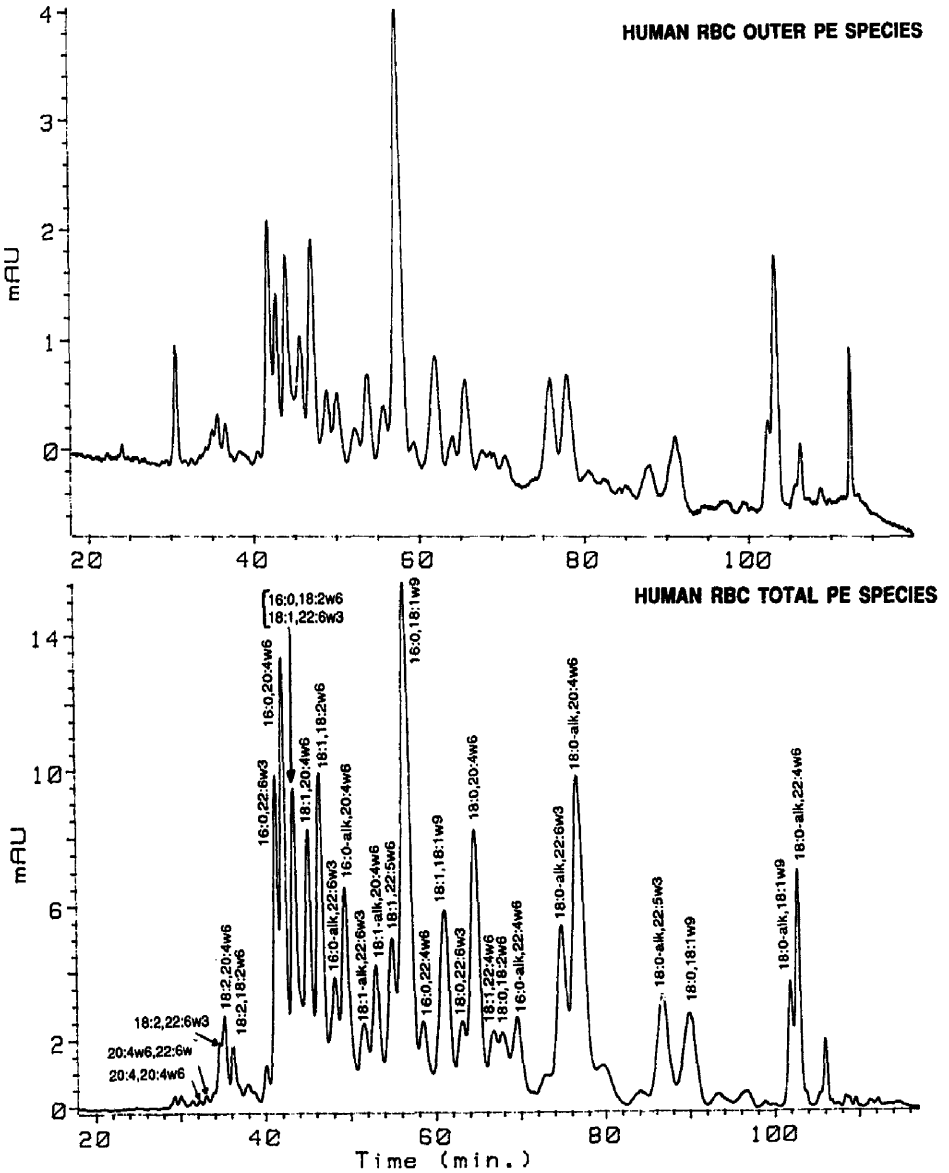

Fig. 2. HPLC analysis of molecular species asymmetry of trinitrophenyl derivatives of human RBC PE. Intact cells were reacted with TNBS, the lipids extracted and purified by TLC and HPLC analysis performed as described in Methods (top panel). The total lipid extracts were reacted with TNBS in organic solution and similarly analysed (bottom panel). An Axxichrom ODS column was used and the mobile phase consisted of 10 mM ammonium acetate (pH 5.0) and methanol. The analysis began with a 5 min isocratic elution at 84% methanol, followed by a linear increase to 87% methanol in 15 min. This was followed by isocratic elution for 70 min and finally by a linear gradient increasing to 93% methanol in 20 min. The flow rate was 1 ml/min and UV detection was at 338 nm. Molecular species were assigned after GC analysis. Alk indicates alkenyl linkage.

extract and thus on the inner leaflet. The main molecular species on the outer leaflet, 16 : 0, 18 : 1w9, accounted for 25% of the outer diacyl species but composed only 17% of the total membrane diacyl species. The sum of the monoene species totalled 30% of the outer PE species but only 20% of the total membrane PE. On the other hand, all the polyunsaturated species accounted for 76% of the total PE species but only 60% of the outer leaflet PE species. The inner leaflet thus appeared to be enriched with polyunsaturated PE species. This mainly involved the *w*–6 species which displayed a more asymmetrical distribution than the *w*–3 species.

TABLE I

Molecular species distribution of trinitrophenylphosphatidylethanolamines in the outer leaflet and total plasma membrane of human RBC [a]

Molecular species [b]	Outer leaflet		Total plasma membrane	
	Diacyl	Alkenyl	Diacyl	Alkenyl
Unidentified	0.05 ± 0.04	–	–	–
Unidentified	0.02 ± 0.02	–	–	–
18:2, 20:5w3 + 18:3, 20:4w6	0.21 ± 0.07	–	0.15 ± 0.02	–
18:2, 18:3w3	0.05 ± 0.04	–	0.19 ± 0.02 *	–
Unidentified	3.70 ± 0.27	–	0.25 ± 0.02 **	–
20:4, 22:6w3 + 16:1, 18:2w6	0.11 ± 0.05	–	0.26 ± 0.02	–
20:4, 20:4w6	0.55 ± 0.06	–	0.29 ± 0.03 **	–
18:2, 22:6w3	–	–	–	–
18:2, 20:4w6 + 16:0, 20:5w3	1.74 ± 0.15	–	2.32 ± 0.09 *	–
di-18:2w6	0.97 ± 0.09	–	1.18 ± 0.05	–
18:2, 22:5w3	0.95 ± 0.07	–	0.90 ± 0.02	–
18:1, 20:5w3	0.33 ± 0.07	–	0.73 ± 0.08 *	–
16:0, 22:6w3	6.18 ± 0.40	7.21 ± 0.37	4.37 ± 0.26 *	5.75 ± 0.20 *
16:0, 20:4w6	4.27 ± 0.28	4.06 ± 0.20	9.68 ± 0.29 **	6.59 ± 0.20 **
16:0, 18:2w6 [c] + 18:1, 22:6w3	7.12 ± 0.26	4.81 ± 0.34	6.50 ± 0.21	4.48 ± 0.15
18:1, 20:4w6	3.85 ± 0.33	5.84 ± 0.40	6.26 ± 0.20 **	3.97 ± 0.13 **
18:1, 18:2w6 [c] + 16:0, 22:5w3	7.01 ± 0.29	5.43 ± 0.34	7.63 ± 0.17	7.41 ± 0.23 **
16:0, 20:3w6	1.63 ± 0.08	–	3.71 ± 0.13 **	–
18:1, 22:5w6	2.30 ± 0.12	–	2.23 ± 0.07	–
16:0, 18:1w9	24.87 ± 0.79	–	16.86 ± 0.13 **	–
16:0, 22:4w6	0.78 ± 0.09	4.99 ± 0.52	1.75 ± 0.10 **	7.03 ± 0.40 *
18:1, 22:5w3	–	1.95 ± 0.23	–	3.11 ± 0.17
di-18:1w9	6.04 ± 0.19	–	6.72 ± 0.16	–
18:0, 22:6w3	1.87 ± 0.08	15.96 ± 0.79	1.78 ± 0.19	11.42 ± 0.56 **
18:0, 20:4w6	4.82 ± 0.25	16.92 ± 0.73	10.08 ± 0.29 **	28.76 ± 0.63 **
18:1, 22:4w6	4.27 ± 0.31	–	4.48 ± 0.13	–
18:0, 18:2w6	–	–	–	–
18:0, 22:5w3	–	6.11 ± 0.45	–	8.79 ± 0.34 **
Unidentified	2.02 ± 0.33	–	1.76 ± 0.30	–
Unidentified	0.40 ± 0.15	–	0.03 ± 0.03	–
Unidentified	1.73 ± 0.18	–	0.80 ± 0.17 *	–
18:0, 18:1w9	6.31 ± 0.21	–	4.58 ± 0.20 **	–
18:0, 22:4w6	0.28 ± 0.09	26.72 ± 1.08	1.17 ± 0.07 **	12.69 ± 0.42 **
Unidentified	0.64 ± 0.20	–	0.96 ± 0.07	–
Unidentified	2.43 ± 0.09	–	1.73 ± 0.06 **	–
Unidentified	0.51 ± 0.09	–	0.34 ± 0.07	–
Unidentified	1.98 ± 0.30	–	0.27 ± 0.06 **	–
Unidentified	0.01 ± 0.01	–	0.04 ± 0.02	–
Totals				
Diacyl + unidentified	71.35 ± 0.55		64.04 ± 0.36 **	
Alkenyl		28.65 ± 0.55		35.96 ± 0.36 **

[a] Values are expressed as the mole% of each PE subclass except for the totals which are mole% of total PE in each membrane fraction. They are the mean and standard error of $n = 19$ determinations. Statistical analysis (ANOVA, Dunnett t) was performed between the outer leaflet and the whole membrane PE species: * $P < 0.01$, ** $P < 0.001$.

[b] Molecular species correspond to the peaks shown in Fig. 2 and were identified by GC analysis. Some of the unidentified peaks are believed to contain alkyl species of PE and represent about 4 and 9% of the PE species in the total membrane and the outer leaflet, respectively.

[c] These species were not found in the alkenyl fractions.

The $w-6$ species composed about 35% and 50% of the outer and total PE species, respectively, whereas the $w-3$ species totalled 25% of the species in each leaflet indicating a more random distribution. Most of the arachidonyl species displayed a highly significant enrichment in the inner leaflet as they represented 31% of the total membrane PE species versus only 18% of the outer PE species. This was true for the diacyl as well as the alkenyl species. The diunsaturated species, 18:1 alk, 20:4$w6$ was an exception as this was at a higher percentage on the outer leaflet. Another exception was the 16:0, 22:6$w3$ species which was slightly enriched on the cell surface.

Highly dipolyunsaturated PE species such as 20:4, 20:4$w6$; 20:4, 22:6$w3$; and 18:3, 20:4$w6$ were also detected in the outer leaflet and total membrane fractions, although they represented a very small proportion of the PE species. It should be noted that it was not possible with this method for molecular species analysis to determine the positions of the fatty acids.

Another interesting point was the preferential localization of the $w-3$ PUFA in the alkenyl species, whereas the $w-6$ PUFA were enriched in the diacyl subclass. About 58% of the $w-3$ PUFA were found in alkenyl species, whereas only 38% of the $w-6$ PUFA were in this subclass for the total membrane. The $w-6$ PUFA distribution on the outer leaflet was similar in that they were more commonly found in the diacyl subclass than alkenyl species. However, the cell surface $w-3$ species displayed a more equal distribution between diacyl and alkenyl subclasses, i.e., 52 vs. 48%.

The PE species asymmetry was further confirmed by fatty acid analysis of the PE labeled in the intact cells and total membrane PE labelled in organic solvent. The proportions of saturates and monoenes were higher in the cell surface pool whereas the proportions of unsaturates was higher in the total membrane fraction (Table II). The ratio of polyunsaturates to monoenes was therefore lower in the outer leaflet (1.86) than in the whole membrane (2.92).

From a consideration of the total amount of PE contained in the erythrocyte total lipid extracts, i.e., 1046 ± 44 nmol/ml packed RBC (mean ± S.E. of $n = 18$ determinations) and the PE species distribution in the outer and inner leaflets, one can calculate the amounts of each PE species on the membrane leaflets. In order to do so, the distribution of PE between the two membrane leaflets must also be known. This distribution has been determined by Verkleij et al. to be 20 and 80 percent on the outside and inside monolayers, respectively [1]. Once the amount of each species is calculated on the outer surface and in the whole cell, one can obtain the amounts on the inner surface by the difference of the two. These values can then be used to estimate the percentages of the total membrane PE for each fatty acid on each membrane leaflet. This calculation is schematically depicted in Fig. 3 and emphasizes the enrichment of monoenes and saturates on the outer leaflet.

TABLE II

Fatty acid composition of phosphatidylethanolamines in the outer leaflet and the total plasma membrane of human RBC [a]

Fatty acids	PE (mole%)	
	outer leaflet	total plasma membrane
Saturates		
16:0	17.65	11.24
18:0	10.30	6.66
Total	27.95	17.90
Monounsaturates		
16:1$w7$	1.25	0.65
18:1$w9/11$	17.04	15.45
20:1$w9/7$	0.40	0.34
22:1$w9$	2.25	0.37
Total	20.94	16.81
$w-6$ PUFA [b]		
18:2$w6$	6.10	5.76
20:2$w6$	0.43	0.31
20:3$w6$	0.82	1.14
20:4$w6$	11.15	21.15
22:4$w6$	5.06	7.28
22:5$w6$	0.55	0.87
Total	24.11	36.51
$w-3$ PUFA		
18:3$w3$	1.22	0.37
20:5$w3$	0.76	1.02
22:5$w3$	3.29	4.09
22:6$w3$	8.38	6.90
Total	13.65	12.38
DMA [c]		
16:0 DMA	4.15	3.00
18:0 DMA	7.19	8.88
18:1 DMA	0.91	4.21
Total	12.25	16.09
PUFA/monoenes	1.86	2.92
U/S [d]	2.14	3.68

[a] The fatty acid distribution was determined by GC analysis of the trinitrophenyl derivatives of PE after TNBS labeling of the intact RBC (outer leaflet) and of the total lipid extract (total plasma membrane) as described in Methods. Data are expressed as mole%.

[b] PUFA indicates polyunsaturated fatty acids.

[c] DMA indicates dimethylacetal derivatives.

[d] U and S indicate unsaturates and saturates, respectively.

Effect of storage on the molecular species asymmetry of PE in the human RBC plasma membrane

In order to evaluate factors that may influence the topological distribution of PE molecular species in the membrane, we compared data obtained with freshly collected RBC with those stored for 24 h at 4°C as either whole blood or in buffer A to which 0.5% BSA was added. Table III showed that there was no statistically significant difference in the distribution of the outer PE species after whole blood was stored for 24 h vs. when processed immediately. Similarly, there was no

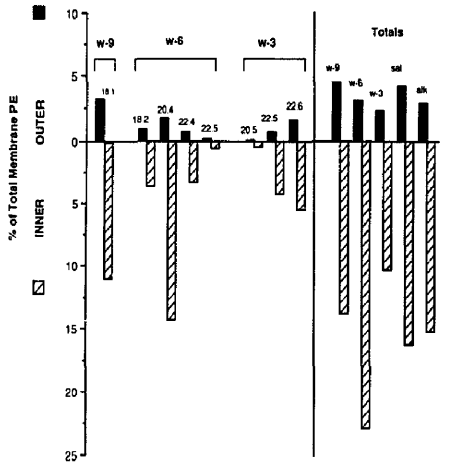

Fig. 3. Fatty acid distributions of inner and outer plasma membrane PE in the human RBC. The absolute amounts of each fatty acid on each leaflet of the RBC plasma membrane was calculated and then expressed as a percentage of total membrane PE (mean of $n = 18$ determinations). Sat indicate saturates and alk the alkenyl moiety in the *sn*-1 position.

difference between the TLE species composition with fresh cells or after 24 h storage as whole blood (data not shown). In contrast, after 24 h of storage in buffer A, we observed a change in the molecular species distribution of the outer leaflet. This was particularly true for the arachidonyl species as it increased on this leaflet concomitantly with a decrease of the monoene species 16:0, 18:1*w*9. Both of these changes are in a direction that serves to decrease the preexisting PE species asymmetry. These alterations became even more pronounced after 48 h storage in buffer A (data not shown).

PS molecular species composition in the human erythrocyte plasma membrane

Fig. 4 showed the HPLC separation of TNP derivatives of PS from the RBC TLE, i.e. whole plasma membrane. As already mentioned, we did not detect any PS labeling when intact cells were treated with TNBS. The amount of PS in the whole membrane was 673 ± 26 mol/ml packed RBC (mean ± S.E. of $n = 6$ determinations). Molecular species were assigned after fatty acid determination by GC analysis of the collected peaks (Table IV). In contrast to PE, the PS fraction was

TABLE III

Effect of storage on the PE molecular species asymmetry in human RBC plasma membrane [a]

Molecular species	Cell surface PE species			Total PE species
	whole blood [b]		washed cells in buffer [c]	
	0 h ($n = 5$)	24 h ($n = 4$)	24 h ($n = 3$)	($n = 5$)
Diacyl				
16:0, 18:1*w*9	19.44 ± 1.30 ***	16.44 ± 0.87 **	15.23 ± 0.54	11.76 ± 0.19
18:0, 18:1	4.96 ± 0.23 ***	4.41 ± 0.39	4.98 ± 0.41	3.16 ± 0.06
18:1, 18:1	4.79 ± 0.28	5.55 ± 0.34	5.44 ± 0.10	4.75 ± 0.09
16:0, 20:4*w*6	2.26 ± 0.10 ***	2.85 ± 0.14 ***	3.44 ± 0.51 *	5.42 ± 0.11
18:0, 20:4	3.15 ± 0.37 ***	3.39 ± 0.28 ***	4.32 ± 0.70 *	7.41 ± 0.31
18:1, 20:4	1.94 ± 0.14 ***	2.14 ± 0.07 ***	2.39 ± 0.22 **	3.74 ± 0.06
16:0, 22:6*w*3	4.58 ± 0.21 *	4.23 ± 0.40	3.78 ± 0.51	3.38 ± 0.05
18:0, 22:6	0.74 ± 0.30	0.54 ± 0.31	0.32 ± 0.32	0.32 ± 0.32
18:1, 22:6	2.56 ± 0.12	2.65 ± 0.03	2.31 ± 0.10	2.06 ± 0.06
Total	70.64 ± 0.25	68.45 ± 0.26	67.35 ± 0.29	64.0 ± 0.09
Alkenyl				
16:0, 20:4*w*6	0.96 ± 0.10 ***	1.26 ± 0.13 ***	1.62 ± 0.18	2.17 ± 0.08
18:0, 20:4	4.23 ± 0.34 ***	5.45 ± 0.64 ***	7.25 ± 0.87	11.21 ± 0.67
18:1, 20:4	1.79 ± 0.11 *	1.70 ± 0.13	1.69 ± 0.16	1.40 ± 0.06
16:0, 22:6*w*3	2.16 ± 0.04	2.39 ± 0.15	2.08 ± 0.09	2.28 ± 0.59
18:0, 22:6	4.88 ± 0.25	5.33 ± 0.42	4.39 ± 0.13	4.40 ± 0.55
18:1, 22:6	1.30 ± 0.05	1.49 ± 0.09	1.55 ± 0.18	1.65 ± 0.14
Total	29.36 ± 0.20	31.55 ± 0.21	32.65 ± 0.25	36.00 ± 0.25

[a] The composition of the major molecular species was obtained after HPLC analysis as described in Methods. Values are expressed as the mole percentage of PE species. Statistical analysis was performed between the outer leaflet and the whole membrane PE species. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; (ANOVA, Dunnett *t*).

[b] Intact RBC were reacted with 2 mM TNBS at 0 °C for 30 min immediately after withdrawal (0 h) or after 24 h storage at 4 °C.

[c] RBC were centrifuged and stored for 24 h in buffer containing 154 mM NaCl, 25 mM glucose, 10 mM Tris–HCl and 0.5% fatty acid-free BSA (pH 7.4) prior to TNBS labeling.

TABLE IV

Phosphatidylserine molecular species composition of the human RBC plasma membrane [a]

Molecular species	Mole %
18:2, 20:4w6	1.11±0.10
16:0, 20:3w6+20:4w6, 22:5w6	0.92±0.10
18:2, 22:6w3+18:2, 22:4w6	1.64±0.07
16:0, 20:4w6+18:1, 22:6w3	5.89±0.31
18:1, 20:4w6	1.72±0.12
16:0, 22:5w3	0.52±0.11
18:0, 20.4w6	61.43±1.13
18:0, 22:6w3	6.54±0.18
18:0, 22:5w3	3.54±0.45
18:0, 22:5w6+18:0, 20:3w6	4.50±0.25
18:0, 18:1w9	4.68±0.23
18:0, 22:4w6	7.51±0.65
PUFA/monoenes	10.53
U/S [b]	1.17

[a] The total lipid extract of human RBC was derivatized with TNBS and the TNP-PS species were separated by TLC, then analyzed by HPLC as described in Methods. The fatty acid composition of each peak separated by HPLC was determined by GC analysis. Values are the mean±S.E. of $n=16$ determinations and are expressed as the mole percentage of total PS.

[b] U and S indicate unsaturates and saturates, respectively.

entirely in the diacyl form. The major molecular species, 18:0, 20:4w6, represented 61% of the PS species. The proportion of polyunsaturates was very high compared to the monoenes representing about 49% and 5% of the fatty acids, respectively. Arachidonic acid was the major PUFA, i.e., about 34% of all the fatty acids. Saturated components were made up principally of stearic acid (44%) and, characteristically for PS, a low proportion of palmitic acid (2%).

Discussion

The work presented here demonstrates that there is a PE molecular species asymmetry in the human RBC plasma membrane. We observed that the outer leaflet was enriched in monoene species, whereas the polyunsaturate species were more concentrated in the membrane interior. These data were obtained using the non-permeant aminophospholipid reagent TNBS. Previous studies with this probe have shown that the aminophospholipids were localized primarily on the inner surface of the RBC membrane, i.e., 80% of the PE and all of the PS [23,25]. This was supported by other approaches using phospholipases [1] or phospholipid exchange proteins [27]. TNBS penetration of the membrane is markedly influenced by the experimental conditions [25] and so they must be carefully chosen if membrane asymmetry is to be studied. The reaction proceeds best at a relatively high pH of 8.0 or more but permeation is restricted at temperature below 10°C. In order to minimize TNBS penetration, we limited the incubation parameters of time (30 min) and temperature (0°C) after choosing pH 8.3 and 2 mM TNBS. Under these non-penetrating conditions, the labeled PE represented about 0.8–0.9% of the total membrane PE. The low amount of labelled PE was still believed to be representative of all the PE species on the outer leaflet

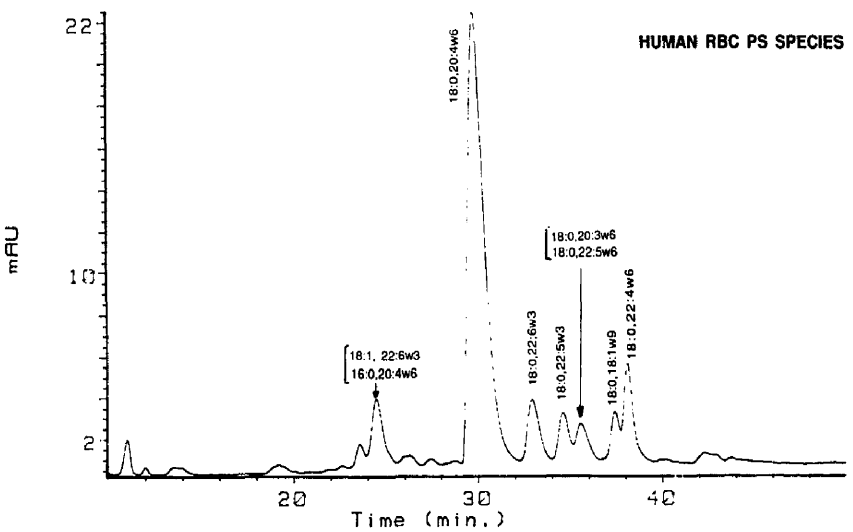

Fig. 4. HPLC analysis of molecular species of trinitrophenyl derivatives of human RBC PS. The RBC total lipid extracts were reacted with TNBS in organic solution and analysed by HPLC as previously described [13]. Detection was by UV at 338 nm. Molecular species were assigned after GC analysis of each peak.

since we demonstrated that all PE species could react with TNBS under these conditions provided that they were accessible to the reagent, i.e. in ghosts or sonicated lipid vesicles. This was consistent with our previous results concerning TNBS reaction with standard PE mixtures [13] as well as the observations of Fontaine and Schroeder [26].

Marinetti and Crain [7] suggested that the PE molecular species localized on the outer surface were of the same distribution as those on the inner surface of the human RBC membrane. Our data do not support their results. However, these authors used different incubation conditions, i.e., 2 mM TNBS for 1 h at 23°C. In addition, the RBC were stored at 4°C for 1 to 2 weeks before TNBS labeling. They mentioned that as the cells aged, they reacted to a greater extent with TNBS indicating penetration into the cells. A similar observation has been made after energy depletion of the RBC [28]. It is likely, therefore, that the higher incubation temperature and extended storage conditions used in their study led either to the permeation of the probe or the loss of membrane species asymmetry and these observations may reconcile their results with those in our study.

Molecular species asymmetry appears to be a general feature of many cell types but may not be found in all cells. Schick et al. [29] found that in the human platelet plasma membrane, the PE molecular species of the cell surface did not differ from that of the inside, except with regard to 18:2*w*6. Similarly, Miljanich et al. [30] found that the fatty acid composition of the inner and outer PE were somewhat similar in the bovine retinal rod outer segment disk membrane with slightly more saturated fatty acids in the inner leaflet. However, an asymmetric distribution of PE molecular species was found in murine synaptosomal membrane [9], trout intestinal brush-border membrane [11], transformed murine fibroblasts [26] and human lymphocytes [10]. In the later case, the degree of asymmetry was not constant but dependent upon the cell cycle. This indicates that the molecular species asymmetry might be an active process related to cell metabolism and maturation. Our results with cells stored in buffer seemed to support this view since a proper energy supply or some other factor present in blood was necessary to maintain species asymmetry. An ATP-dependent 'translocase' may be involved in the creation and maintenance of PE species asymmetry since it has been proposed that this system is responsible for aminophospholipid class asymmetry [31]. If so, it would appear that the system must possess a substrate selectivity for particular molecular species.

In this study, the *w*–3, *w*–6 and *w*–9 fatty acid distribution in PE subclasses of RBC was described. Alkyl species were not identified by our analytical procedures, however, this subclass represents only a small proportion (2%) of the human erythrocyte PE [50] and they may account for some of the unidentified peaks. The proportions of diacyl and alkenyl subclasses obtained for the total lipid extract are in good agreement with the results of others [32]. We found that the *w*–3 PUFA were preferentially localized in the alkenyl-PE, whereas the *w*–6 PUFA and the *w*–9 unsaturates were enriched in the diacyl subclass. This particular feature was conserved in the outer as well as the inner leaflet. The *w*–3 species on the outer leaflet were more randomly distributed between the diacyl and alkenyl subclasses. It is well known that the ethanolamine plasmalogens contain relatively high levels of PUFA [33]. The preference of the *w*–3 PUFA, particularly 22:6*w*3, for this subclass might be the consequence of the prior incorporation of the PUFA in the precursor alkylacyl-PE. The selectivity of the Δ1 desaturase for the alkylacyl-PE containing 22:6*w*3 in the *sn*-2 position has been demonstrated as well as a high turnover of these species [34]. A rapid turnover of the docosahexaenoate species in the ether-containing PE has been also reported in rat brain [35] and in Ehrlich ascite cells [36]. These mechanisms might explain the preference of *w*–3 fatty acids, particularly 22:6*w*3, for the alkenyl-PE. In addition, recent dietary studies have shown a preferential increase of the *w*–3 fatty acids in the alkenyl-PE in rat erythrocytes [17], human platelets [37] and a macrophage-like cell line [38]. Dipolyunsaturated species were observed in the diacyl subclass, e.g., 20:4*w*6, 20:4*w*6; 20:4*w*6, 22:6*w*3; and 18:3, 20:4*w*6. Despite their small proportions, these unusual species may play an important role in membrane function due to their high metabolic turnover [39] as well as their unusual physical properties.

The phenomenon of molecular species asymmetry raises questions about the metabolic consequences of this localization. The preferential localization of the arachidonyl-PE species on the cell interior is important since phospholipids are considered to be the source of free arachidonic for eicosanoid synthesis. More generally, if both phospholipid class and molecular species asymmetry are considered, taken together with the fact that the acidic lipids found on the interior leaflet contain a high level of 20:4*w*6 as well as other PUFAs, there is a high concentration of these PUFAs on the interior leaflet. Even though no cyclooxygenase has been demonstrated in erythrocytes, there is a report of lipoxygenase activity in mammalian erythrocytes [40]; these observations are of obvious relevance to other cell types with more active eicosanoid biosynthesis and function. The asymmetric localization of arachidonic acid in the inner leaflet of the plasma membrane might provide for a more efficient coupling between the release and transformation of arachidonic acid.

The preferential localization in the inner leaflet of polyunsaturated PE molecular species may be expected to influence membrane physical properties [4,41]. Studies with spin-labeled phospholipids suggested an asym-

metric fluidity in erythrocyte membranes with a more rigid outer layer and a more fluid inner layer [42]. This difference in the fluidity was attributed to the asymmetric distribution of phospholipid classes in the RBC membrane as well as to interactions between lipids and proteins. It should be added that the asymmetric distribution of the PE molecular species may also contribute to this difference. Also, studies have shown the importance of molecular species composition in determining erythrocyte shape, deformability and membrane stability [43,44]. Increasing the amounts of disaturated-PC induced formation of echinocytes, whereas introduction of dilinoleoyl-PC led to formation of stomatocytes. An asymmetric distribution of molecular species may participate in the maintenance of the biconcave disk shape. Furthermore the fluidizing properties of polyunsaturated species might be amplified if these species are non-randomly distributed in the membrane. Their influence on the physical state should be important for the regulation of the biological activities of membrane proteins associated with these microenvironments [4,41].

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