Phospholipid Localization in the Plasma Membrane of Friend Erythroleukemic Cells and Mouse Erythrocytes†

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ABSTRACT: The distribution of phospholipids over outer and inner layers of the plasma membranes of Friend erythroleukemic cells (Friend cells) and mature mouse erythrocytes has been determined. The various techniques which have been applied to establish the phospholipid localization include the following: phospholipase A_2, phospholipase C, and sphingomyelinase C treatment, fluorescamine labeling of phosphatidylethanolamine, and a phosphatidylcholine transfer protein mediated exchange procedure. The data obtained with these different techniques were found to be in good agreement with each other. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol were found to be distributed symmetrically over both layers of the plasma membrane of Friend cells. In contrast, sphingomyelin was found to be enriched in the outer layer of the membrane (80–85%), and phosphatidylserine appeared to be present mainly in the inner layer (80–90%). From these results, it was calculated that the outer and inner layers accounted for 46% and 54%, respectively, of the total phospholipid complement of that membrane. Analogous studies on the plasma membrane of mature mouse erythrocytes showed that the transbilayer distribution of the total phospholipid mass appeared to be the same as in the plasma membrane of the Friend cell, namely, 46% and 54% in outer and inner layers, respectively. The outer layer of this membrane contains 57% of the phosphatidylcholine, 20% of the phosphatidylethanolamine, 85% of the sphingomyelin, and 42% of the phosphatidylinositol, and none of the phosphatidylserine was present. A comparison of the results obtained with the Friend cell and with the mature mouse erythrocyte may indicate that the typical asymmetric distribution of phospholipids, found in all erythrocyte membranes, is partially apparent already at an early stage of erythropoiesis.

The distribution of phospholipids over the inner and outer layers of biological membranes has been extensively studied during the last decade, and one of the best examples of a membrane in which an asymmetric phospholipid distribution has been unequivocally established is the erythrocyte membrane (Op den Kamp, 1979; Etemadi, 1980; Roelofsen, 1982).

An important problem related to phospholipid asymmetry in erythrocytes is that of its biogenesis. Indeed, the erythrocyte represents the end product of a complex differentiation pathway which requires several days to be completed. During this process, which starts in the bone marrow, proliferating pluripotential stem cells become committed to erythropoiesis and evolute through a succession of developmental stages (proerythroblast, basophilic erythroblast, polychromatophilic erythroblast) to the normoblast stage. Then, upon extrusion of the nucleus, a reticulocyte is formed which enters into the blood circulation, where it completes its maturation to the final erythrocyte stage (Harrison, 1976).

One may thus ask what the phospholipid distribution in the plasma membrane of the erythroblast is and whether this distribution is similar (or not) to that existing in the mature erythrocyte. If it is different, it is essential to investigate which mechanisms are underlying the change(s) in phospholipid distribution and at which stage(s) of the differentiation pathway these modifications occur. We have attempted to answer the first of these questions using Friend erythroleukemic cells (Friend cells) as an experimental system. Friend cells are erythroid cells, derived from susceptible mouse spleens infected with the Friend virus complex (Zajdela, 1962), which are blocked at an early stage of their normal differentiation pathway, presumably between the BFU-E (burst-forming unit erythroid) and CFU-E (colony-forming unit erythroid) stages (Harrison, 1976; Troxler et al., 1980; Kost et al., 1979). These cells, which only proliferate under normal growth conditions, can be induced to differentiate at least up to a normoblast-like stage by a variety of chemicals, among which dimethyl sulfoxide is most commonly used (Friend et al., 1971). The morphological and biochemical characteristics of their developmental program have been intensively investigated and in many respects closely resemble those of the normal erythropoietic pathway (Reuben et al., 1980; Marks & Rifkind, 1978).

We have recently reported the isolation and characterization of plasma membranes from Friend cells (Rawyler et al., 1982, 1983) and have shown that sphingomyelin was asymmetrically distributed in the plasma membrane (Rawyler et al., 1983). In this paper, we present a comparative study of the phospholipid distribution in the Friend cell plasma membrane and in the mouse erythrocyte membrane. Several independent techniques were used, namely, nonlytic treatments with phospholipase A_2, phospholipase C, and sphingomyelinase C, chemical labeling of aminophospholipids with fluorescamine, and protein-catalyzed phosphatidylcholine exchange.

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Materials and Methods

Cells. Friend cells (derived from clone 745 A) were cultured, washed, and counted as described elsewhere (Rawyler et al., 1983; Storm et al., 1982). Resuspension of cells was achieved by adding a small amount of buffer to the cell pellet and using the up/down pumping action of a Pasteur pipet, taking care to avoid frothing; then, more buffer was added, and the cells were centrifuged.

Mouse blood was drawn from DBA/2J or BALB/c mice and collected on acid citrate/dextrose. Erythrocytes were washed 3 times in 0.9% NaCl containing 5 mM glucose, resuspended in the desired buffer (see below), and used immediately.

Phospholipases. Bee venom phospholipase A_2 (EC 3.1.1.4) was purified from crude bee venom (Shipolini et al., 1971). Phospholipase C from *Bacillus cereus* was prepared according to Little (1981). Sphingomyelinase C from *Staphylococcus aureus* was purified as described by Zwaal et al. (1975). All enzymes were stored at −20 °C in buffered (pH 7.5) 50% glycerol.

Phosphatidylcholine Transfer Protein. This protein was prepared from beef liver and purified according to Westerman et al. (1983). Before use, the required amount of transfer protein was freed of glycerol by dialysis against adequate buffer and concentrated to the original volume over poly(ethylene glycol) flakes (Calbiochem).

Phospholipase Treatments. All incubations were carried out at 37 °C in a shaking water bath. Friend cells were resuspended in buffer A (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM Tricine, pH 7.4). Incubations were started by the addition of enzymes (10 IU of phospholipase C, 2 IU of phospholipase A_2, or 0.5 IU of sphingomyelinase C per 10×10^6 cells). For each time point, samples taken contained 60×10^6 cells (corresponding to about 750 nmol of lipid phosphorus) in a volume of 6 mL.

Mouse erythrocytes were also resuspended in buffer A. Incubations were started by addition of enzymes (40 IU of phospholipase C, 4–10 IU of phospholipase A_2, or 3 IU of sphingomyelinase C per 150 μL of packed cells). For each time point, samples contained 150 μL of packed cells (corresponding to about 650–700 nmol of lipid phosphorus) in a volume of 6 mL.

Incubations were terminated by mixing the samples (6 mL) with 1 mL of 35 mM ethylenediaminetetraacetic acid (EDTA) in buffer A (for phospholipase A_2 and sphingomyelinase C) or with 1 mL of 30 mM EDTA *plus* 30 mM *o*-phenanthroline in buffer A (for phospholipase C).

Fluorescamine Labeling of Aminophospholipids. Friend cells were treated with increasing amounts of fluorescamine at low temperature (0–4 °C) exactly as described elsewhere (Rawyler et al., 1984). Mouse erythrocytes were labeled at 15–30 °C by using the same procedure, except that the labeled erythrocytes were diluted 5-fold with 6 mM glycylglycine in buffer A (pH 8) as quenching agent.

Incubation with Phosphatidylcholine Transfer Protein. Friend cells were washed 3 times with Hank's balanced salt solution. Red blood cells were washed 3 times with buffer B (150 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM EDTA, 5 mM glucose, and 20 mM Tricine, pH 7.4). Rat liver microsomal membranes were isolated from Wistar rats which were injected intraperitoneally with 30 μCi of [*methyl*-^{14}C]-choline (Kamp & Wirtz, 1974). Before use, they were sonicated in buffer B at 0 °C using a Branson sonifier (60 W) until the suspension became opalescent (usually 2 min). Remaining large particles were spun down at 8000*g* for 10 min. The supernatant was used for the incubations. Cells, microsomes, and phosphatidylcholine (PC) transfer protein [purified as described by Westerman et al. (1983)] were preequilibrated at 37 °C for 5 min.

All incubations were carried out at 37 °C in a thermostated room on a clinical blood rotator at 4 rpm. Incubations were started by the addition of microsomes to a cell suspension containing 4×10^7 Friend cells/mL or 20–30% (v/v) red blood cells and 3–4 μM PC transfer protein in the appropriate buffer. The molar ratio of microsomal to cell PC was about 1:2. As a control, equal amounts of cells and microsomes without PC transfer protein were incubated under the same conditions. At timed intervals, samples were taken and diluted 10-fold in appropriate buffer at 37 °C. Cells were isolated by centrifugation at 2500*g* for 5 min, and the supernatants were used to measure the extent of cell lysis. The cells were washed another 2 times with buffer to remove microsomal membranes before lipid extraction. The extent of PC exchange was calculated as described elsewhere (van Meer & Op den Kamp, 1982).

Control of Cell Lysis. The extent of cell lysis induced by the above-mentioned treatments was determined by monitoring the hemoglobin release from erythrocytes and by assay of the lactate dehydrogenase from Friend cells (Rawyler et al., 1983).

Lipid Extractions. Friend cell pellets were extracted according to Reed et al. (1960) after resuspension in 0.5 mL of buffer A. Samples incubated in the presence of phospholipases were resuspended in 0.5 mL of buffer A containing the appropriate inhibitors (see above) to ensure that no additional hydrolysis would occur during extraction. Mouse erythrocytes were extracted by the method of Rose & Oklander (1965), using the same precautions. All lipid extracts were subjected to a Folch wash (1957).

Analytical Procedures. The phospholipid composition of each lipid extract was determined by two-dimensional thin-layer chromatography (Broekhuyse, 1969), followed by phosphorus determination of the individual phospholipid spots (Böttcher et al., 1961). Radioactivity was measured in 299 TM emulsifier scintillator solution from Packard by using a Packard-PRIAS-Tricarb scintillation counter.

Results

For sake of clarity, the results concerning Friend cells and those concerning mouse erythrocytes will be dealt with in two separate sections.

Friend Cells

Friend cells, unlike (mouse) erythrocytes, possess a considerable amount of intracellular membranes. Consequently, the phospholipid complement of the Friend cell plasma membrane must be known before any comparison can be made with the erythrocyte membrane. Using two independent techniques, viz., nonlytic treatments of Friend cells with sphingomyelinase C (Rawyler et al., 1983) and fluorescamine (Rawyler et al., 1984), we have recently established using the calculation procedure of Chap et al. (1977) that the plasma membrane of Friend cells accounts for 38.3–38.5% of the total cellular phospholipids. From this result and the known compositions of the phospholipid fractions extracted from whole cells and isolated plasma membranes (Rawyler et al., 1983), the relative abundance of each phospholipid class in the plasma membrane of Friend cells could be easily calculated (Rawyler et al., 1984; see also Table I). It is interesting to note that some phospholipids are mainly (lysophosphatidylcholine and sphingomyelin) or even exclusively (phosphatidylserine) present in the plasma membrane of these cells, whereas phosphatidylinositol

Table I: Relative Abundance of Each Phospholipid Class in the Plasma Membrane of Friend Cells

phospholipid	compn (mol %) in cells[a]	compn (mol %) in plasma membranes[a]	amount of each phospholipid class in plasma membrane: % total phospholipid	amount of each phospholipid class in plasma membrane: % of phospholipid class
phosphatidylcholine	59.9	55.2	21.2	35.8
phosphatidylethanolamine	18.5	16.5	6.3	34.3
sphingomyelin	5.6	10.2	4.0	70.7
phosphatidylinositol	8.3	5.9	2.3	27.6
phosphatidylserine	4.0	10.6	4.1	103.0
lysophosphatidylcholine	0.7	1.4	0.5	77.6
diphosphatidylglycerol	3.0	nd		
	100.0[b]	100.0[b]	38.4[b]	

[a] Taken from Rawyler et al. (1983). [b] Total.

Table II: Availability of Phospholipids to Different Reagents in Intact Friend Cells[a]

probe	line	PC	PE	PS	PI	SPH
sphingomyelinase C	a					56–60[c]
	b					3.1–3.4
phospholipase A_2	a	16–17	17–19		13–14	
	b	9.6–10.2	3.2–3.5		1.1–1.2	
phospholipase C	a	15–16	12–14	15–20		
	b	9.0–9.6	2.2–2.6	0.6–0.8		
phosphatidylcholine transfer protein	a	16–18				
	b	9.6–10.8				
fluorescamine[b]	a		16–18	9–10		
	b		3.0–3.3	0.4		

[a] Data are expressed as percent of each phospholipid class (lines a) and as percent of total cell phospholipid (lines b). Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin. [b] Only the first pools, comprising the aminophospholipids in the outer monolayer of the plasma membrane (see text). [c] See also Rawyler et al. (1983).

appears to be predominantly located in intracellular membrane systems.

(*A*) *Treatment with Phospholipases.* Phospholipase A_2 treatment of whole Friend cells resulted in the hydrolysis of 16–17% of the phosphatidylcholine and 17–19% of the phosphatidylethanolamine and caused a maximum cell lysis of only 5–7%. Phosphatidylinositol was hydrolyzed to a comparable extent, although a plateau was less clearly defined in this case. A semilogarithmic plot of the hydrolysis data (not shown) indicated that 13–14% of the cellular phosphatidylinositol was degraded under these conditions. On the other hand, the phosphatidylserine content of Friend cells remained unaltered under these conditions.

When Friend cells were treated with phospholipase C or with sphingomyelinase C, approximately 15–16% of the phosphatidylcholine, 12–14% of the phosphatidylethanolamine, 5–7% of the phosphatidylserine *plus* phosphatidylinositol, and 56% of the sphingomyelin were degraded. Since pure phospholipase C from *B. cereus* is essentially unable to degrade phosphatidylinositol (Roelofsen, 1982), the degradation of 5–7% of the anionic phospholipids should account for the hydrolysis of phosphatidylserine only, which thus corresponds to 15–20% of the total amount of this phospholipid present in the cell.

Since the integrity of Friend cells was essentially conserved during these treatments, it is likely that the observed hydrolysis patterns reflect the degradation of the phospholipids present in the outer monolayer of the plasma membrane. The plateau values for hydrolysis, which are already reached after 20 min of incubation, did not change during a subsequent incubation of 2 h, suggesting that—with the possible exception of phosphatidylinositol—all phospholipids present in the outer monolayer may have been degraded and that no transbilayer movement of phospholipid molecules from the inner to the outer face of the plasma membrane occurs in the time scale of the experiments.

(*B*) *Fluorescamine Labeling of Aminophospholipids.* We have recently investigated the conditions under which the permeant probe fluorescamine can be used to completely localize phosphatidylethanolamine in intact Friend cells (Rawyler et al., 1984). A semilogarithmic plot of the analytical data revealed that the labeling patterns of phosphatidylethanolamine are constituted of three pools, representing the phosphatidylethanolamine in the outer and inner monolayers of the plasma membrane as well as that in the intracellular membrane systems. The relative sizes of these pools have been determined to be 16–18%, 16–20%, and 64–66% of the total cell phosphatidylethanolamine, respectively (Rawyler et al., 1984). About 40% of the phosphatidylethanolamine is present in the plasmalogen form, but the distribution over the different pools of phosphatidylethanolamine is not known. From further studies, it appeared that the labeling of phosphatidylserine is about 6 times less efficient than that of phosphatidylethanolamine. However, two pools could be observed. The first comprised 9–10% of the phosphatidylserine and should be attributed to the outer monolayer of the plasma membrane, while the second, slow-reacting pool should represent the progressive labeling of the inner phosphatidylserine.

(*C*) *Protein-Catalyzed Transfer of Phosphatidylcholine.* Friend cells were incubated with [^{14}C]PC-labeled rat liver microsomal membranes and PC-specific exchange protein, essentially as described under Materials and Methods. The transfer of [^{14}C]PC from microsomes to Friend cells was determined and used to calculate (van Meer & Op den Kamp, 1982) the amount of PC in the outer leaflet of the Friend cell which is accessible for the exchange process. About 15% of the total cell PC appeared to be exchangeable during a 4-h incubation period, and no additional exchange, at least not at a measurable rate, occurred afterward.

(*D*) *Transbilayer Distribution of Phospholipids in the Plasma Membrane of Friend Cells.* Table II summarizes the results of all the above experiments concerning Friend cells. The amount of each phospholipid, detectable with the various techniques under nonlytic conditions, is expressed as the percent of the cellular content of each phospholipid class and

Table III: Relative Amounts of Friend Cell Plasma Membrane Phospholipids Present in the Outer Monolayer[a]

probe	PC (%)	PE (%)	PS (%)	PI (%)	SPH (%)
sphingomyelinase C					79–85[b]
phospholipase A_2	45–47	50–55		47–51	
phospholipase C	42–45	35–41	15–20		
phosphatidylcholine transfer protein	45–50				
fluorescamine		47–52	9–10		

[a] Data are expressed as the percent of the total amount of each phospholipid class present in the plasma membrane. These values are calculated by using the relative abundance data of Table I, column 5, and the data on the relative availability of each phospholipid class for exogenous probes (Table II, lines a). For abbreviations, see Table II. [b] See also Rawyler et al. (1983).

as the percent of the total cell phospholipid. All techniques give comparable values for each phospholipid. It can be calculated, by adding up the figures given in Table II, lines b, that the outer monolayer of the plasma membrane accounts for 15.8–19.7% of the total cell phospholipid, averaging at 17.8%. The plasma membrane contains 38.4% of the total cell phospholipid (Table I). Thus, it can be concluded that the inner monolayer of the plasma membrane is only slightly enriched in phospholipid (54%) compared to the outer monolayer (46%). From the data given in Tables I (column 5) and II (lines a), one can calculate for each phospholipid class which fraction of its total amount in the plasma membrane is present in the outer monolayer. Results of such calculations are summarized in Table III. It can be seen that the two major phospholipids of the plasma membrane, phosphatidylcholine and phosphatidylethanolamine, are essentially symmetrically distributed over the two monolayers. A similar arrangement can be tentatively proposed for phosphatidylinositol, be it that the experimental evidence is less conclusive. On the other hand, the plasma membrane is strongly asymmetric with respect to the distribution of sphingomyelin (80–85% outside) and phosphatidylserine (10–20% outside).

Mouse Erythrocytes

(*A*) *Treatment with Phospholipases.* DBA/2J mouse erythrocytes were incubated at 37 °C in the presence of either phospholipase A_2 or sphingomyelinase C. Sphingomyelinase C was able to degrade sphingomyelin, and a well-defined plateau in the hydrolysis profile was reached after 85% of this phospholipid was hydrolyzed. The same result was obtained when BALB/c mouse erythrocytes were used. In both cases, there was a maximum of 3% hemolysis. This shows that the sphingomyelinase C treatment did not damage the membrane integrity. Moreover, when open ghosts were submitted to such treatment, sphingomyelin was completely degraded (not shown) within 20 min. These data can thus be interpreted in terms of an asymmetric distribution of sphingomyelin in the mouse erythrocyte. The outer and inner monolayers would thus account for 85% and 15%, respectively, of the total sphingomyelin.

When DBA/2J mouse erythrocytes were submitted to phospholipase A_2 treatment, the degradation of glycerophospholipids included a maximum of 20% phosphatidylethanolamine and about 12% of the anionic phospholipids. Surprisingly, only 30% of the phosphatidylcholine could be hydrolyzed in these incubations, during which hemolysis was limited to 5–8%. Additional degradation of phosphatidylcholine was possible by increasing the incubation time, but considerable hemolysis occurred in that case. On the other hand, more than 90% of the glycerophospholipids could be degraded in 20 min when open ghosts were treated with

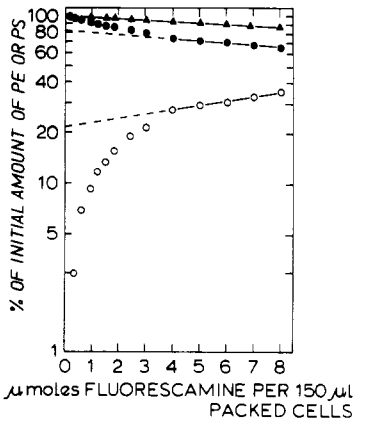

FIGURE 1: Concentration-dependent labeling of aminophospholipids by fluorescamine in mouse erythrocytes. The decrease in phosphatidylethanolamine (●) and the increase in its fluorescamine derivative (○) are calculated by using the amount of phosphatidylcholine as an internal standard and expressed as the percent of the initial amount present. Phosphatidylserine (▲) labeling is also expressed as the percent of its initial amount. Cell lysis was always less than 5% for all fluorescamine concentrations.

corresponding amounts of phospholipase A_2 (not shown). One can calculate from these data that a maximum of 29% of the total phospholipids (thus including sphingomyelin) can be degraded in intact erythrocytes. It is highly unlikely that the outer monolayer would contain only this relatively small amount, as such a distribution would create a large gap with respect to the inner monolayer. Therefore, an additional series of experiments with phospholipase A_2 was performed, namely, an incubation with a mixture of the *Naja naja* phospholipase A_2 with bee venom phospholipase A_2, a combination of bee venom phospholipase A_2 with sphingomyelinase C, and finally, a combination of phospholipase A_2 treatment with the removal of split products using serum albumin as described by Haest et al. (1981). All these treatments were equally inefficient in that no further hydrolysis of phospholipids was obtained. Attempts to use the phospholipase C as a tool to localize phospholipids failed, since phospholipase C and *B. cereus* did not induce any significant hydrolysis, whereas that from *Clostridium welchii* appeared to cause extensive hemolysis. Both these results are identical with those observed with human erythrocytes (Verkleij et al., 1973; Zwaal et al., 1975).

(*B*) *Experiments with Phosphatidylcholine Transfer Protein.* Monolayer experiments have shown (F. A. Kuypers, unpublished results) that the phosphatidylcholine transfer protein is able to exchange its substrate up to the collapse pressure of the monomolecular film. This property is attractive in view of the equivocal data obtained for the localization of phosphatidylcholine using phospholipase A_2. Indeed, incubation of mouse erythrocytes with [^{14}C]PC-labeled microsomes and PC-specific exchange protein resulted in exchange of 50–60% of the total PC present in the membrane. The residual PC does not become accessible for the exchange protein in prolonged incubations, up to 8 h, which strongly indicates, as discussed before (van Meer et al., 1980), that 50–60% of the total PC is present in the outer layer of the membrane, the remainder being at the inside.

(*C*) *Labeling with Fluorescamine.* Phospholipase A_2 treatment of mouse erythrocytes showed that about 20% of phosphatidylethanolamine could be assigned to the outer monolayer. Although a well-defined hydrolysis plateau was obtained for this phospholipid (and also for anionic phospholipids), the enzymatic approach was not completely satisfactory. Chemical labeling of aminophospholipids with fluorescamine was therefore undertaken. Figure 1 presents

Table IV: Phospholipid Localization in the Mouse Erythrocyte Membrane[a]

technique used	% of each phospholipid class in the outer monolayer				
	PC	PE	PS	PI	SPH
sphingomyelinase C					85
phospholipase A_2	(30)[b]	20	0	42	
phosphatidylcholine transfer protein	50–60				
fluorescamine		20	0		

[a] The phospholipid composition (mol %) of mouse erythrocytes is 45.4% phosphatidylcholine, 24.3% phosphatidylethanolamine, 11.5% sphingomyelin, 10.8% phosphatidylserine, 4.1% phosphatidylinositol, and 4% lysophosphatidylcholine. For abbreviations, see Table II. [b] Incomplete degradation.

the concentration-dependent labeling of phosphatidylethanolamine and of phosphatidylserine in DBA/2J mouse erythrocytes, expressed in a semilogarithmic way. Both phospholipids became labeled, but with a much lower efficiency than in Friend cells [compare Rawyler et al. (1984)]. Whether this is due to an intrinsically lower reactivity of the mouse erythrocyte aminophospholipids toward fluorescamine or to some steric hindrance requiring higher concentrations of label cannot be decided now. However, it can be seen that phosphatidylserine became labeled as one slow-reacting pool, extrapolating to 98–100%. Although detectable on the chromatogram, the fluorescamine derivative of phosphatidylserine was present in such low amounts that a reliable phosphorus determination could not be carried out. The labeling pattern of phosphatidylethanolamine was obviously biphasic. The extrapolation of the second linear component of residual PE yielded a value of 80–82%, which was confirmed by a complementary value of 20–21% for the corresponding fluorescamine–PE derivative. These data can be interpreted as indicating that the outer monolayer of the mouse erythrocyte membrane is essentially depleted of phosphatidylserine and contains only 20% of the phosphatidylethanolamine, which is in perfect agreement with the results obtained from the phospholipase A_2 experiments.

Table IV summarizes the results of all the above experiments concerning mouse erythrocytes. The outer monolayer would contain 50–60% of the phosphatidylcholine, 20% of the phosphatidylethanolamine, and 85% of the sphingomyelin. In addition, since no phosphatidylserine is present in this outer monolayer, it can be extrapolated from the results of the phospholipase A_2 incubation that the 12% of anionic phospholipids degradable by phospholipase A_2 under nonlytic conditions represents the hydrolysis of phosphatidylinositol only. This would lead to the tentative conclusion that 58% of the phosphatidylinositol molecules are localized in the inner monolayer. On the basis of these data and the phospholipid composition of mouse erythrocytes (see legend to Table IV), one can calculate that the outer and the inner monolayers contain, respectively, 46% and 54% of the total phospholipids.

Discussion

Studies on phospholipid distribution in the plasma membrane of mammalian cells are hampered by the presence of intracellular membranes. Various methodological approaches have been used to overcome this difficulty. Most studies employed plasma membrane derivatives such as phagolysosomes (Sandra & Pagano, 1978) or enveloped viruses (Rothman et al., 1976; van Meer et al., 1981) instead of the actual cellular plasma membrane. Advantages and limitations of these indirect techniques have been amply discussed elsewhere (Op den Kamp, 1979), but it may be useful to recall that the phospholipid distribution obtained on a given derivative of the plasma membrane should not be automatically extended to the parent structure, namely, the plasma membrane of the cell. Other studies involved controlled treatment(s) of the cells followed by isolation and analysis of the plasma membrane from control and treated cells (Fontaine & Schroeder, 1979). The major drawback in this case consists of numerous, time-consuming plasma membrane isolations.

Most of these problems can be eliminated if one knows the percentage of cellular phospholipid present in the plasma membrane (relative abundance). Once the exact composition of the phospholipids extracted from both isolated plasma membranes and whole cells has been established, the relative abundance in the plasma membrane can be calculated for each phospholipid class. The method of Chap et al. (1977) is particularly well suited for this purpose. With this method, the relative abundances of protein, cholesterol, and of total phospholipid in the plasma membrane of Friend cells have been recently determined (Rawyler et al., 1983). These calculations were extended here to each individual phospholipid class (Table I). The most salient feature was obviously the absolute confinement of phosphatidylserine within the plasma membrane of Friend cells. Data obtained by using a completely independent technique (Rawyler et al., 1984) fully supported this observation.

Coherent results were obtained between the various techniques used to localize phospholipids in the plasma membrane of Friend cells (Table III) as well as in the mouse erythrocyte membrane (Table IV). Measurements made under nonequilibrium conditions (e.g., fluorescamine labeling of aminophospholipids) were confirmed by data obtained from equilibrium conditions (e.g., phospholipases and exchange experiments). Ranges of values were necessary to express the phospholipid distribution in the plasma membrane of Friend cells (Table III). This was due to the methodological approach chosen and to the way calculations have to be made. However, there is a remarkable homogeneity in the results when they are expressed on a total phospholipid basis (Table II, lines b).

An asymmetric phospholipid distribution has already been established in a number of erythrocyte species, including those of man, pig, rabbit, rat, chicken, and sheep (Op den Kamp, 1979). In all cases, the choline-containing phospholipids were localized preferentially outside, whereas phosphatidylethanolamine and the anionic phospholipids were found mainly in the inner monolayer. The present results indicate that the mouse erythrocyte is no exception in this respect. Its phospholipid distribution conforms to the usual trend, be it that the PC distribution is somewhat less asymmetric as found in other erythrocytes.

The failure of phospholipase A_2 (either from *N. naja* or bee venom) to degrade all of its substrates present in the outer monolayer of an erythrocyte has been well documented for the human red cell (Roelofsen, 1982; Verkleij et al., 1973; Zwaal et al., 1975). It has been argued (Roelofsen et al., 1980) that the action of the phospholipase A_2 ceases as a consequence of an increase in lateral surface pressure in the outer monolayer of the intact cell, which is caused by the generation of split products (lysophospholipids and free fatty acids) herein. Hydrolysis of sphingomyelin by sphingomyelinase C is believed to have the opposite effect, namely, a decrease in lateral surface pressure (Roelofsen, 1982; Roelofsen et al., 1980). Including this enzyme in incubations with phospholipase A_2 therefore enables the latter phospholipase to degrade its substrates in the outer monolayer to completion (Roelofsen, 1982; Verkleij et al., 1973; Zwaal et al., 1975). However, the combined

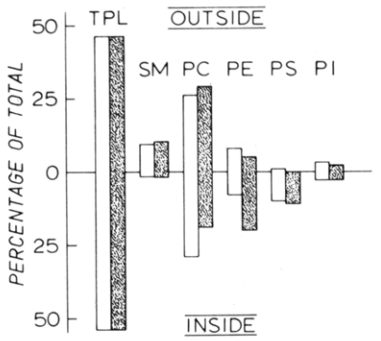

FIGURE 2: Phospholipid composition and transbilayer distribution in plasma membranes of Friend erythroleukemic cells (open bars) and mature mouse erythrocytes (solid bars). Abbreviations: TPL, total phospholipid; SM, sphingomyelin; PC, PE, PS, and PI, phosphatidylcholine, -ethanolamine, -serine, and -inositol, respectively.

action of phospholipase A_2 *plus* sphingomyelinase C (the latter enzyme being added 10 min after the addition of phospholipase A_2) applied on mouse erythrocytes did not increase the extent of glycerophospholipid hydrolysis during a 1-h incubation period. This may be due to the relatively low sphingomyelin content of the mouse erythrocyte when compared to that in the human red cell, making the sphingomyelinase C induced decrease in lateral surface pressure too limited to give the phospholipase A_2 an additional chance. Another possibility to achieve a decrease in packing pressure in the outer monolayer may be found in removal of the hydrolysis products with bovine serum albumin (Haest et al., 1981). As mentioned above, even this approach did not result in any further degradation of the PC in this membrane. As yet, we have no explanation for this remarkable finding.

Friend erythroleukemic cells are transformed cells, and it is not known in detail if transformation affects the phospholipid composition and distribution of the plasma membrane. Therefore, a direct comparison of Friend cells and proerythroblasts may hardly be justified. Nevertheless, one may speculate that in case the plasma membrane of Friend cells and its normal, nontransformed, counterpart have a comparable phospholipid distribution, several changes must occur on the distribution of phospholipids in the plasma membrane during erythropoiesis (Figure 2). Phosphatidylcholine and phosphatidylethanolamine must concentrate, respectively, in the outer and the inner monolayer of the plasma membrane. Also, a rearrangement of phosphatidylserine has to take place, be it to a limited extent. Phosphatidylinositol and sphingomyelin, however, are already distributed in the Friend cell plasma membrane in a way which is found also for the erythrocyte membrane. The latter observation once more emphasizes the very special place this sphingomyelin appears to occupy among the phospholipids in the red cell membrane. In contrast to (some of) the glycerophospholipids, the most pronounced asymmetric distribution of sphingomyelin is altered neither in chemically modified normal human erythrocytes (Haest et al., 1978) nor in sickled erythrocytes (Lubin et al., 1981). Furthermore, it was recently found (Boegheim et al., 1983) that, unlike phosphatidylcholine and phosphatidylethanolamine, the sphingomyelin in the (human) red cell membrane possess a high degree of asymmetry in the composition of its molecular species at either side of the membrane, indicating the virtual absence of transbilayer movements of this phospholipid. In this context, it is relevant to recall that in plasma membranes of various other cell types, e.g., BHK (van Meer et al., 1981) and MDBK cells (Rothman et al., 1976), sphingomyelin adopts another, less asymmetric, transbilayer distribution.

From the above changes we observed in composition and transbilayer distribution of the glycerophospholipids, two sets of questions can be addressed. The first is related to the temporal sequence of such events. Are they precocious or late? Do they occur together for all phospholipid classes concerned, or does each phospholipid class undergo its own change independently of the other classes? The second group of questions concerns the nature of the molecular mechanisms through which these changes occur.

The time course of the alterations in phospholipid distribution could be related to the increase in the amount of preexisting membrane proteins such as spectrin and/or to the appearance of newly synthesized membrane proteins such as band 3 (Pfeffer & Redman, 1981). Accordingly, the major changes should occur when the degree of organization of these membrane proteins into the plasma membrane reaches its final state. In this case, phospholipid reorientation would be the result of a progressive adaptation of the lipid matrix to the specific lipid–protein interactions within the differentiating plasma membrane.

Later and more rapid changes in phospholipid distribution may occur during the enucleation of the normoblast. Geiduschek & Singer (1979) reported that spectrin as well as certain membrane glycoproteins underwent large diffusional movements in opposite directions within the plasma membrane, spectrin becoming concentrated in the enucleated portion of the cell. Lipid–protein interactions such as those of phosphatidylserine and phosphatidylethanolamine with spectrin (Haest et al., 1978) might then favor an increase in the inside:outside distribution ratio of these phospholipids.

Alternatively, further adjustments in phospholipid distribution may occur at an even later stage, during which the enucleated cell (reticulocyte) enters into the blood stream. In this case, the change(s) in the chemical composition of the surrounding medium (e.g., type and concentration of electrolytes) might be actively involved in the modification of the phospholipid distribution (McLaughlin & Harary, 1974).

The use of differentiating Friend cells is now expected to provide more information about the temporal sequence of the events linked to the biogenesis of the phospholipid asymmetry in mouse erythrocytes and about the identity of the molecular mechanism(s) by which the phospholipid distribution is altered during differentiation.

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Interactions between Core Histones and Chromatin at Physiological Ionic Strength†

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ABSTRACT: Addition of core histones to chromatin or chromatin core particles at physiological ionic strength results in soluble nucleohistone complexes when polyglutamic acid is included in the sample. The interaction between nucleosomes and added core histones is strong enough to inhibit nucleosome formation on a closed circular DNA in the same solution. Complexes consisting of core particles and core histones run as discrete nucleoprotein particles on polyacrylamide gels. Consistent with the electrophoretic properties of these particles, protein cross-linking with dimethyl suberimidate indicates that added core histones are bound as excess octamers. Histones in the excess octamers do not exchange with nucleosomal core histones at an ionic strength of 0.1 M and can be selectively removed from core particles by incubating the complexes in a solution containing sufficient DNA. Under conditions where added histones are confined to the surface of chromatin, the excess histones are mobile and can migrate onto a contiguous extension of naked DNA and form nucleosomes.

During chromatin replication, newly made histones enter the nucleus and rapidly form nucleosomes on nascent DNA (Seale, 1978; Cremisi, 1979; Laskey & Earnshaw, 1980). Although in vitro studies have shown that, under certain conditions, histones spontaneously fold DNA into nucleosomes (McGhee & Felsenfeld, 1980; Laskey & Earnshaw, 1980), it has also been found that the core histones interact rather strongly with chromatin (Voordouw & Eisenberg, 1978; Stein, 1979). Because there is a large amount of chromatin in the nucleus, relative to nascent DNA, it is of interest to know how nucleosome assembly is influenced by the presence of chromatin. It is obvious that chromatin does not inhibit nucleosome assembly in vivo but it is not really clear why this is the case (Jackson et al., 1976).

Histone–chromatin interactions have been difficult to study because the nucleohistone complexes formed are insoluble at physiological ionic strength. Added histones induce precipi-

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