TOPOLOGY AND DYNAMICS OF PHOSPHOLIPIDS IN MEMBRANES

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Received 18 November 1980

1. Introduction

The present lecture summarizes some studies on the transbilayer distribution of phospholipids in biomembranes, and in addition deals with recent investigations on the translocation of phospholipids between the outer and inner compartment. The topology of phospholipids has been studied mainly by chemical labelling, enzymatic modification with phospholipases, and through the use of exchange on transfer proteins (fig.1). In principle the approach is simple, namely a comparison of the action of these tools on intact and open membranes, but many pitfalls can be made. Merits and limitations of the various methods have been discussed in detail in a recent review by Op den Kamp [1]. In order to obtain unambiguous results the complete action of the impermeable probe should leave the membrane structure intact and not cause phospholipid redistribution. Experience has shown that it is often difficult to meet all prerequisites and for several membranes information about phospholipid topology is either incomplete or contradictory. However, a non-random distribution of phospholipids in the erythrocyte membrane has been established now by many investigators and the overall view is rather uniform.

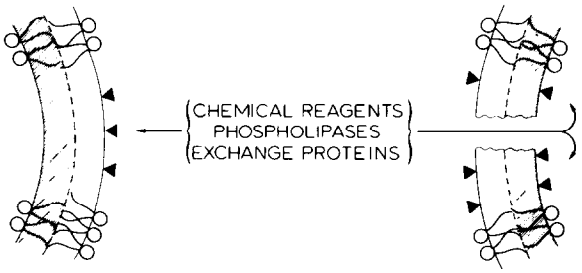

Fig.1. Determination of the topology of phospholipids in biomembranes. Experiments are performed on intact erythrocytes and on open ghosts.

2. Topology of phospholipids in the red cell membrane

First evidence that the amino-phospholipids are localized primarily on the inner surface of the red cell membrane was obtained by using non-penetrating chemical reagents. Bretscher [2] showed that a relatively impermeant agent like formyl methionylsulphone-methylphosphate does not react with phospholipids in intact cells, whereas both phosphatidylethanolamine and phosphatidylserine react in open membranes. Using trinitrobenzene sulphonate Gordesky and Marinetti [3] found that this reagent did not label phosphatidylserine in intact cells and that only part of phosphatidylethanolamine reacted.

Using a particular combination of phospholipases [4,5] a non-lytic degradation of nearly 50% of the phospholipids in intact human erythrocytes was obtained (fig.2). In as much as this enzymatic hydrolysis causes a complete degradation of all phospholipid classes in open membranes the attack on the intact cells is considered to be limited to the constituents present in the outer layer of the membrane. This phospholipid fraction comprises in the human erythrocyte: about 80% of sphingomyelin, 75% of phosphatidylcholine and 20% of phosphatidylethanolamine; apparently the aminophosphoglycerides, in particular phosphatidylserine, are concentrated at the cytoplasmic side of the red cell membrane. Supporting evidence for this asymmetric distribution was obtained by studies on the action of a phospholipase on the inside of resealed membranes [6] and experiments on sealed inside-out vesicles [7], as well as by experiments with exchange proteins (discussed below). Although quantitative differences exist a similar distribution of choline- and amino-containing phospholipids has been found in erythrocytes of various mammalian species [8]. Within one phospholipid class, e.g., phosphatidylcholine, no significant differences appear to exist in

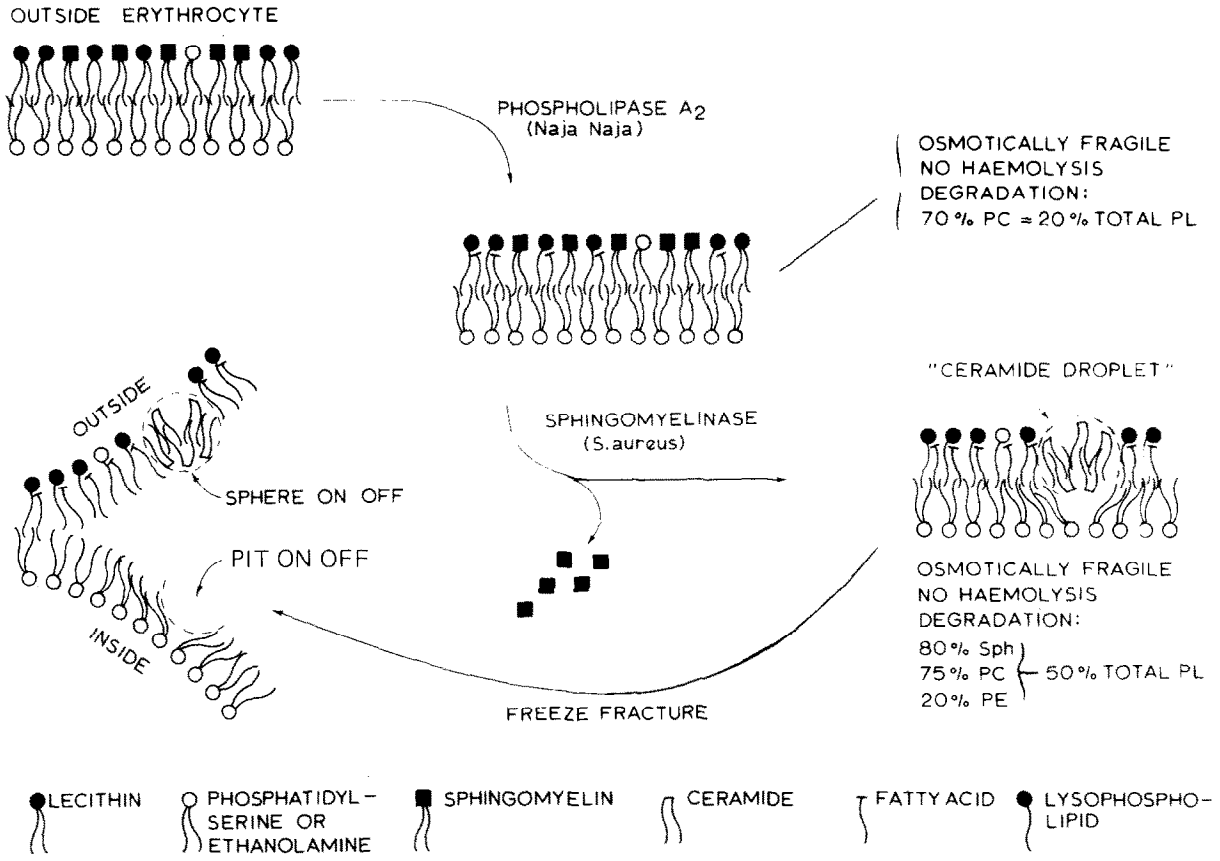

Fig.2. Schematic representation of the non-lytic action of two phospholipases on intact human erythrocytes. Action of these phospholipases on open ghosts produces 100% phospholipid hydrolysis.

the fatty acid composition between the two pools.

The physiological significance of the phospholipid asymmetry of red blood cells is still subject to speculation. It is of interest to note that the choline-containing phospholipids are known to arrange spontaneously in a stable bilayer configuration, this in contrast to phosphatidylethanolamine. Furthermore, the asymmetric arrangement of phospholipids in blood cells may be related to blood coagulation. In the plasma membrane of platelets a phospholipid asymmetry was detected which is comparable to the distribution found in red cells [9]. The exterior surface is devoid of those phospholipids which promote the conversion of prothrombin and a redistribution of phosphatidylserine over the platelet membrane may be involved in the control of coagulation [10].

The non-random distribution of phospholipids in the erythrocyte membrane is probably linked to the asymmetric disposition of membrane proteins. Suggestions have been forwarded by several laboratories that spectrin, which is located at the cytoplasmic side of the red cell membrane, may be preferentially associated with the amino-phospholipids [11–14].

3. Preservation of bilayer structure after phospholipase action on the red cell membrane

A prerequisite for studies on the phospholipid topology is that the basic structure of the membrane is not significantly disturbed by the phospholipase attack. Because the formation of lysophospholipids, free fatty acids, diacylglycerols and ceramides may be expected to modify the bilayer organization, it was rather surprising that with these enzymes clear-cut results could be obtained on the asymmetric distribution of phospholipids over the inner and outer region of the erythrocyte membranes. Several approaches

have given evidence for a preservation of the gross membrane organization and lipid-bilayer configuration after phospholipid degradation in red cells.

(*i*) In a study combining the use of phospholipases and freeze-etch electron microscopy, Verkleij et al. [4] observed that freeze-fracturing of phospholipase-treated erythrocytes is still possible, which in fact indicates the preservation of a lipid bilayer. Furthermore, it was observed that the action of a sphingomyelinase on intact red cells produced the formation of small spheres (75 Å and 200 Å diameter) on the outer fracture face with corresponding pits on the inner fracture face. It was suggested that these spheres represent small droplets of ceramides produced by the breakdown of sphingomyelin in the outer monolayer of the membrane. On the other hand freeze-etching did not reveal an accumulation of lysophospholipids and fatty acid produced by phospholipase A_2 action on intact cells. It was concluded that a gross disorganization of the membrane did not occur although cells treated with phospholipases revealed morphological changes and an increased osmotic fragility relative to control cells [4].

(*ii*) Wilbers et al. [15] studied the influence of phospholipase action on the permeability of the erythrocyte membrane. After hydrolysis of up to 65% of phosphatidylcholine or sphingomyelin, non-electrolyte diffusion in the intact red cells was unaffected. It was concluded that the permeability barrier of the lipid core is not significantly altered after degradation of the phospholipids present in the outer membrane layer.

(*iii*) ^{31}P NMR studies on organized systems of pure lipids have demonstrated the possibility to detect the occurrence of bilayer, hexagonal and micellar configurations of phospholipids in model systems, and this technique could be applied successfully to biological membranes (for a recent review compare Cullis and De Kruyff [16]). The erythrocyte membrane exhibits ^{31}P NMR spectra which are consistent with the vast majority of the lipid being in the bilayer configuration. After treatment of intact cells with phospholipase A_2 alone (fig.3) or in combination with sphingomyelinase, the spectra of ghosts obtained from such erythrocytes revealed that the residual phospholipids and lysophospholipids remain organized in a bilayer arrangement [17]. Even after a nearly complete enzymatic degradation of the phospholipids in erythrocyte ghosts no signal was observed indicating isotropic ^{31}P motion despite the fact that the hydrolysis products individually prefer non-bilayer configurations.

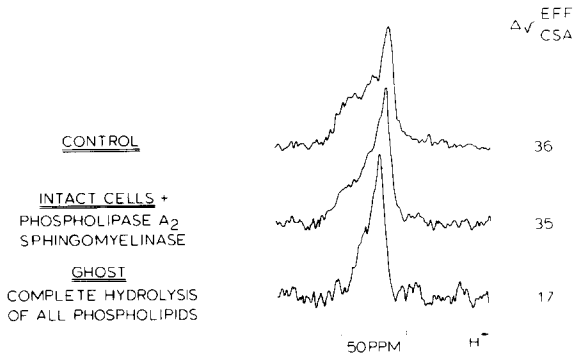

Fig.3. ^{31}P NMR spectra of phospholipase-treated erythrocytes and ghosts. The spectra indicate that after phospholipid hydrolysis the gross bilayer arrangement of the membrane is maintained [17].

Apparently, the frame work of the erythrocyte membrane is highly stable and the maintenance of a lipid-bilayer structure after phospholipid hydrolysis may be governed in part by the organization of membrane proteins. In addition cholesterol which is abundant in erythrocytes can stabilize a bilayer orientation of lysolecithin [18,19]. Furthermore, phospholipase A_2 produces equimolar amounts of monoacylphosphoglyceride and free fatty acid and it was recently shown by Jain et al. [20] that lysophosphatidylcholine associates with fatty acids in an aqueous phase so as to form a bilayer-type of organization. Whereas the individual components form micelles when dispersed in aqueous phase, a mixture of fatty acid and lysophosphatidylcholine exhibits birefringence, and differential scanning calorimetry showed phase transition properties indicative for interaction between the acyl chains of the components. ^{31}P NMR studies revealed spectra typical for a bilayer type of organization; freeze-fracturing demonstrated the existence of vesicular structures (1000–6000 Å diameter) with fracture planes similar to those present in phosphatidylcholine dispersions. Also the hemolytic action of lysophosphatidylcholine appeared to be retarded by the presence of fatty acids. These observations suggest that

lysophosphatidylcholine and free fatty acid tend to interact to give a structure that resembles that of diacylphosphatidylcholine, thus explaining that a bilayer configuration is maintained even after extensive phospholipid hydrolysis. Under such conditions exogenous phospholipases do not have access to phospholipids in the interior layer and act exclusively on the constituents present in the outer membrane layer. This presumption is correct only when the rate of transbilayer movement of phospholipid is slow when compared to the reaction time required for phospholipase action.

4. Translocation of phosphatidylcholine in the red cell membrane

4.1. *Studies with phospholipases*

With the aid of phospholipases, evidence was given that a sidedness exists in phosphatidylcholine renewal in the red cell membrane and strong indications were obtained that a slow translocation of this phospholipid may occur across the membrane. In mature mammalian erythrocyte two mechanisms appear to play a major role in phospholipid renewal:

(*i*) Exchange of phospholipids between serum lipoproteins and the cell membranes;

(*ii*) Enzymatic incorporation of fatty acids into lysophospholipids, which are either formed in the membrane or supplied by the serum.

Studies of Renooy et al. [8,21,22] showed that two metabolically different pools contribute to the turnover of phosphatidylcholine in the red cell membrane and showed that the two pathways occur in different compartments of the membrane (fig.4). Incubation of rat erythrocytes in ^{32}P-labelled plasma results in a labelling of phosphatidylcholine of the membrane; the specific radioactivity of the lecithin fraction which is degradable by a non-lytic phospholipase treatment of the cells outranks the activity of the non-susceptible fraction. The most logical explanation implies that the exchange process between cell and environment involves the phospholipid molecules of the exterior membrane layer. Incubation of erythrocytes with radioactive fatty acids labels in particular phosphatidylcholine. Treatment of the intact labeled cells with phospholipases showed that the specific radioactivity is higher in the non-degradable pool when compared with the non-lytic hydrolysis of the susceptible pool of phosphatidylcholine in the membrane indicating that fatty acid incorporation occurs at the inside of the membrane. The conversion of labelled lysophosphatidylcholine into phosphatidylcholine revealed a similar labelling pattern over both pools [8].

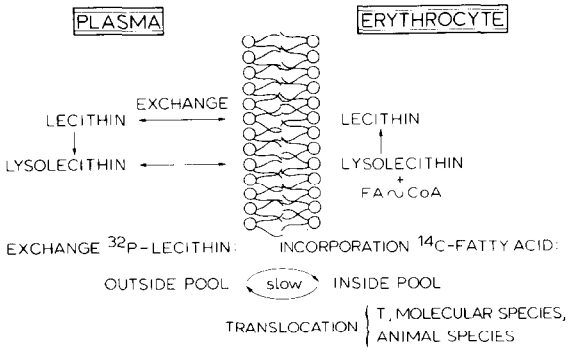

Fig.4. Metabolic asymmetry and transbilayer movement of phosphatidylcholine in the erythrocyte membrane. Exchange with serum lipoproteins occurs at the outer layer whereas transacylation of monoacylphosphatidylcholine (lysolecithin) is located at the cytoplasmic side.

Apparently, the exchange of phosphatidylcholine and the incorporation of fatty acid into lysophosphatidylcholine occur in two compartments, which can be distinguished by a non-lytic phospholipase degradation. In addition this approach enabled Renooy et al. [22,23] to detect a slow and temperature-dependent transmembrane movement of phosphatidylcholine in the rat erythrocyte membrane. After labelling via exchange with ^{32}P-labelled plasma, the specific radioactivity of membrane phosphatidylcholine susceptible to phospholipase A_2 action appeared to decrease with time. By contrast, it was observed that after labelling of phosphatidylcholine by incorporation of ^{14}C-labelled fatty acid the radioactivity of phospholipid which can be hydrolysed by phospholipases at the outside of the cells increased with incubation time. A transbilayer exchange between the phosphatidylcholine interior and exterior compartments was more directly demonstrated by first labelling rat erythrocyte phospholipids, followed by removal of the ^{32}P-labelled plasma and subsequent reincubation of the cells in a buffer; the total radioactivity of the cells remained constant, but the specific radioactivity of the degradable fraction in the intact cells decreased with time, whereas a corresponding increase was observed in the phosphatidylcholine fraction which is not susceptible toward phospholipase action. A reversed pattern of redistribution of label was observed after incorporation of ^{14}C-labelled fatty acids into phosphatidylcholine by a transacylation of lysophosphatidylcholine. The redistribution of both labels

appears to occur in phosphatidylcholine of intact rat erythrocytes with an average half-time rate of 4–5 h at 37°C. This transmembrane movement is much slower than that more recently reported by Hirata and Axelrod [24] on phosphatidylcholine synthesized in rat erythrocytes by methylation of phosphatidylethanolamine. This process of stepwise methylation involves one enzyme on the cytoplasmic side acting on phosphatidylethanolamine and a second enzyme at the external surface, which methylates phosphatidyl-*N*-monomethyl ethanolamine to phosphatidylcholine. These authors proposed a mechanism for an enzyme-mediated bilayer movement of phosphatidylcholine. It is likely that the slow translocation discussed above links the exchange reaction occurring at the outer region with the transacylation of lysophosphatidylcholine at the cytoplasmic side.

Renooy and Van Golde [25] concluded that the exchange reaction with plasma and the outer layer of the erythrocyte is mainly responsible for the renewal of (poly)unsaturated species of phosphatidylcholine of the erythrocyte and that transacylation activity is primarily directed towards the formation of disaturated phosphatidylcholines at the inside of the membrane. Transmembrane movement appears to be faster for the more unsaturated phosphatidylcholine species than for the disaturated molecules and the translocation rate is temperature dependent [22,26]. It is of interest to note that the rate of the translocation process differs between erythrocytes of various mammalian species; by contrast to rat erythrocytes, in human erythrocytes no significant transmembrane movement of phosphatidylcholine was observed at 37°C, but was detectable at 45°C [8].

4.2. *Studies with phospholipid exchange proteins*

Phospholipid exchange proteins can be considered to be attractive tools for studying asymmetry and transbilayer movement of phospholipids, because membrane perturbation probably is rather minimal. Exchange of phospholipid from red cells was reported to occur with a non-purified supernatant fraction from a rat liver homogenate [27]. However, in early studies using purified preparations the specific proteins were found to be unable to catalyze the exchange of phosphatidylcholine in intact cells [28–30]. On the other hand, an exchange protein was found to be active on resealed rat erythrocyte ghosts and Bloj and Zilversmit [30] demonstrated the asymmetric disposition and transbilayer movement of phosphatidylcholine in these membrane structures in fair agreement with data obtained by phospholipase action on intact cells. Kramer and Branton [31] observed a retention of lipid asymmetry in rat erythrocyte membranes isolated on polylysine-coated beads. Using a phosphatidylcholine-specific exchange protein it was observed that in membranes where the cytoplasmic surface is exposed about 36% of the total phosphatidylcholine is readily available for exchange while the remaining 64% is exchangeable at a much slower rate. These values agree very well with results obtained by previous investigators.

Recently, two laboratories reported on the use of purified phospholipid exchange proteins for the determination of transbilayer distribution and mobility of phospholipids in intact red cells. Van Meer et al. [32] observed that the well-characterized protein from beef liver, which specifically transfers phosphatidylcholine, acts on erythrocytes provided that a sufficiently high concentration of this protein is present. When human erythrocytes and microsomes containing ^{14}C-labelled phosphatidylcholine were incubated in the presence of exchange proteins about 75% of the phosphatidylcholine was exchanged after 2 h, and no additional exchange was observed (fig.5) in the subsequent 2 h of incubation. The results indicated that 75% of phosphatidylcholine is present in the outer layer; this value is in excellent agreement with the data previously obtained with phospholipases. Furthermore, these experiments confirm the earlier observations obtained with the phospholipase degradation technique that at 37°C in human erythrocytes the transbilayer movement of phosphatidylcholine is not detectable [8]. In rat erythrocytes about 50–60% of phosphatidylcholine was readily available for the exchange protein which value is in fair agreement with the value (62%)

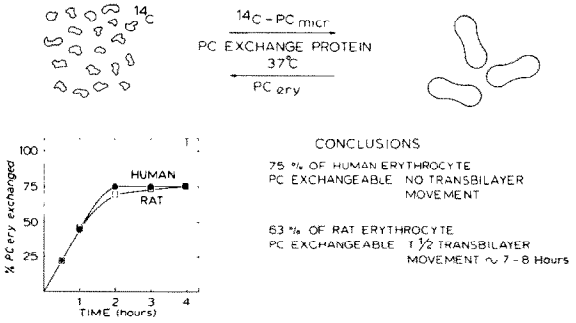

Fig.5. Exchange of phosphatidylcholine between intact erythrocytes and microsomes by an exchange protein [32].

detected at the outer region by means of phospholipase action. In contrast to human erythrocytes in rat erythrocytes the exchange does not stop after 2 h incubation, but a second pool of phosphatidylcholine appears to become available for exchange at a much lower rate. This biphasic behaviour allowed to calculate a half-life for equilibration of phosphatidylcholine between the two compartments of the same order as previously obtained by phospholipase degradation of labelled rat erythrocytes [22].

Further confirmation and extension of the data on asymmetry and translocation of phospholipids in rat erythrocytes was independently provided by Crain and Zilversmit [33] who used a non-specific (or universal) exchange protein from beef liver. The transfer of labelled phosphatidylcholine from erythrocytes to unilamellar vesicles revealed a rapid phase involving 63% of the pool whereas 37% is transferred slowly, giving a similar rate of transbilayer exchange as mentioned above. Furthermore, this study showed that all of the sphingomyelin and very little of the phosphatidylethanolamine is localized in the outer layer, thus corroborating the conclusion of Renooy et al. [22] obtained with phospholipase degradation. With regard to phosphatidylethanolamine localization it should be recalled that this phospholipid becomes accessible to phospholipase A_2 after previous degradation of sphingomyelin by sphingomyelinase [6,22].

5. Replacement of membrane phospholipid by exchange protein

In order to establish the possible relationship between chemical make-up and function of lipids a systematic introduction of structural variations in one single species of membrane constituents is a method of choice. In this respect significant progress was made, e.g., by alteration of the fatty acid composition in microorganisms. However, in intact mammalian membranes such studies have been more difficult. Although it is possible to alter by diet the fatty acid pattern of, e.g., red cell phospholipids [34], homeostatic mechanism operating in vivo will limit the chemical changes so as to keep the overall physical properties of the membrane within certain limits. Exchange proteins offer several opportunities to modify in a well-controlled manner the membrane lipid composition. In a recent study of Lange et al. [35] phosphatidylcholine from intact rat erythrocytes was replaced by a series of synthetic species after incubation with lipid vesicles in the presence of a phosphatidylcholine specific exchange protein. Under the chosen conditions neither the total lipid content, nor the phospholipid–cholesterol ratio or ratio of different phospholipid classes was altered and the single variation concerned the increase of the relative content of one particular phosphatidylcholine species. For instance after incubation with vesicles of labelled (dipalmitoyl)phosphatidylcholine the uptake was followed in the erythrocyte and the increase in the content of this species was compensated for by a loss at random of other molecular species of phosphatidylcholine with different fatty acid combinations. This process did not effect the integrity of the cells until about 25% of the native phosphatidylcholine has been replaced by (dipalmitoyl)phosphatidylcholine, at which point cell lysis occurred. At the onset of hemolysis of the rat erythrocytes the content of disaturated species was increased from about 35% to 50% of the phosphatidylcholine population. Similar results were obtained with other saturated phosphatidylcholines although some differences were noted between the onset of hemolysis and chain-length of the fatty acid constituents. On the other hand, the introduction of (dioleoyl)phosphatidylcholine up to levels of 60% of the native erythrocyte phosphatidylcholine did not result in rapid hemolysis. These studies show that the fatty acid composition of phosphatidylcholine is an important parameters in the maintenance of membrane integrity of the red cell. Furthermore, it is intriguing that certain modifications in the proportions of the individual molecular species of a membrane phospholipid cause red cell lysis, which does not occur after extensive phospholipid degradation of the membrane outer layer. During the initial stages of exchange no differences between treated and control cells can be observed by scanning electron microscopy, but echinocytes were formed when about 10–15% of the erythrocyte phosphatidylcholine had been replaced by the disaturated species (fig.6).

Freeze-fracture electron micrographs show the evaginations of the membrane in detail [35]. When exchange was continued spherocytes were formed (fig.6). Preliminary studies indicate that in rat erythrocytes the replacement of native phosphatidylcholine by an increased content of disaturated species is accompanied by an increased potassium permeability and an altered osmotic fragility which precedes cell lysis. Further studies are necessary to explain the various observations in molecular terms.

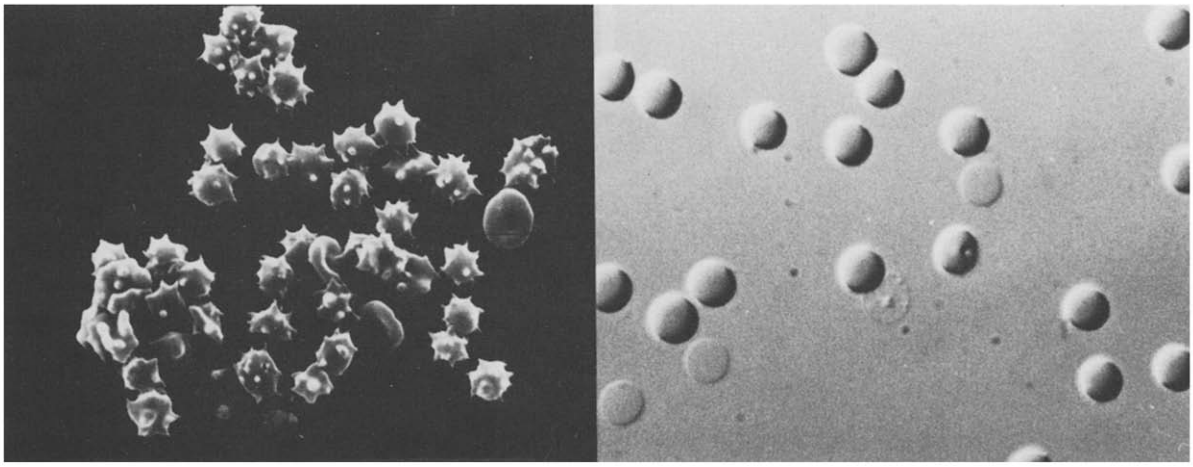

Fig.6. Left: Scanning electron micrograph of rat erythrocytes in which 15% of native phosphatidylcholine has been replaced by synthetic (dipalmitoyl)phosphatidylcholine. Right: Interference light micrograph of rat erythrocytes in which 25% of native phosphatidylcholine has been replaced by (dipalmitoyl)phosphatidylcholine.

6. Phospholipid organization in microsomal membranes

Several laboratories have studied the phospholipid topology of microsomal membranes, but to date it is not possible to depict a conclusive model. Using phospholipases several research groups arrived at contradictory or inconclusive results [36–40]. It is not impossible that treatment of microsomal membranes with phospholipases leads to membrane rearrangement thereby facilitating transbilayer movement of certain phospholipid classes. The present status of research in this area clearly illustrates the difficulties one can encounter with phospholipase as tools for phospholipid localization and detailed investigations will be necessary to understand and resolve the existing discrepancies.

In as much as phospholipid exchange proteins are supposed to induce less membrane perturbation it was expected that more unambiguous information could be obtained with these tools, but attempts to localize phospholipids in microsomes by this method also failed. The major phospholipid of rat liver microsomes appear to be completely available for exchange, indicating that transbilayer movement of phospholipids is a relatively fast process in this membrane [40–42]. Studying the disposition of phosphatidylcholine with the aid of the exchange protein specific for this phospholipid, Van den Besselaar et al. [40] observed that at 25°C and 37°C rat liver microsomal phosphatidylcholine was completely and rapidly available for replacement; a 8°C and 0°C complex exchange kinetics were observed, indicating the presence of several pools of phosphatidylcholine in this membrane.

Transmembrane movement of phospholipids in the microsomal membrane can be considered to have a physiological function because biosynthesis of phospholipids appears to occur on the cytoplasmic side of the membrane [43,44] while secretion occurs via the lumen of the endoplasmic reticulum [45]. It is of interest to note that a rapid transmembrane movement of phospholipids was observed in *Bacillus megaterium* [46], which appears to be independent of lipid synthesis and sources of metabolic energy [47].

A dynamic behaviour of phospholipids in liver microsomal membranes was demonstrated also by ^{31}P NMR studies, which technique yields information on the microscopic structures adopted by phospholipid molecules. In contrast to the erythrocyte membrane, microsomal preparations were found independently in two laboratories [48,49] to give rise to ^{31}P NMR spectra which indicate isotropic motion for some fraction of the membrane phospholipids at 37°C. A portion of the phospholipids may exist in a non-bilayer arrangement which could involve inverted micellar structures being in rapid exchange with the bulk bilayer lipid. At lower temperatures an increasing fraction of the phospholipid adopts the bilayer configuration and it is possible that a correlation exists between the observed behaviour of phosphatidylcholine towards

exchange protein at various temperatures. Although the isotropic motion observed by ^{31}P NMR does not arise from microsomal tumbling, the possibility that an enhanced lateral diffusion around the small microsomal vesicles contributes to this phenomenon is not precluded [50]. On the other hand, the dynamic formation of inverted micelles in a lipid bilayer as depicted by Cullis and De Kruijff [16], can account for the isotropic behaviour and the transmembrane movement detected with exchange proteins. In model systems non-bilayer configuration can be induced by effects on lipid parameters [16], but also membrane proteins, e.g., cytochrome *P*-450 have been proposed to be capable of producing isotropic motion of phospholipid [48]. It should be mentioned that bile salt-depleted rat liver microsomes revealed similar results indicating that bile salts are not responsible for phospholipid mobility in this membrane [51]. As mentioned above, a transbilayer movement of phospholipid can be considered as a physiological necessity of metabolically active membranes. Phospholipid transport across the membrane does not preclude the existence of an unequal distribution of phospholipid classes over the two sides of the microsomal membrane. The transverse distribution of phosphatidylcholine can in principle be investigated by ^{13}C NMR. After feeding rats a diet containing [*N*-*Me*$_3$-^{13}C]choline the inside–outside distribution of phosphatidylcholine in sarcoplasmic reticulum was determined with the shift reagent $DyCl_3$ [52], but for technical reasons this approach failed for the endoplasmic reticulum.

7. Inducation of transbilayer movement of phospholipid in model systems

At present the molecular mechanism of either a slow or a rapid translocation of phospholipids across biological interfaces is not understood. In this respect experiments with membrane model systems may be helpful. The classic experiments of Kornberg and McConnell [53] already demonstrated that the translocation (flip–flop) of a spin-labelled phosphatidylcholine across the bilayer of pure lipid vesicles is a slow process. Subsequent studies by several groups using a number of techniques confirmed that transbilayer exchange of phospholipids in unilamellar vesicles is extremely slow [1,45]. Even incorporation of lysophosphatidylcholine – a compound which can be envisaged to cause some disturbance of the bilayer arrangement – appears not to be translocated at detectable speed [54,55]. On the other hand it was found, during the past years, that a transbilayer movement of phospholipids can be induced in bilayer vesicles in different ways, including:

(*i*) Introducing different physical properties between the outer and the inner monolayer;
(*ii*) Insertion of bilayer-spanning proteins;
(*iii*) Triggering of the formation of non-bilayer arrangements in the lipid barrier.

(*i*) Phospholipase D was used by De Kruijff and Baken [56] to convert phosphatidylcholine to phosphatidic acid in unilamellar vesicles which preserved their barrier properties. Using paramagnetic ions the transbilayer distribution of both phospholipids was determined by ^{31}P NMR during enzyme action; phosphatidic acid formed in the outer monolayer was found to be translocated to the inner monolayer with a half-time of $\leqslant$30–40 min at 25°C, and an appropriately equal number of phosphatidylcholine molecules apparently moved from the inner to the outer layer at a comparable rate. These results suggest that in biomembranes which have an asymmetric distribution of metabolic pathways, enzymatically introduced differences in physical properties of lipids (e.g., charge and size of polar headgroups) may cause transbilayer movement. At the same time these experiments indicate that care is necessary when using phospholipases for the determination of phospholipid topology in biomembranes (compare also [57,58]).

Induction of differences in the composition and consequently the physical properties of the apolar moieties of the phospholipids between the two halves of the bilayer also presents a driving force for transbilayer exchange of lipids. De Kruijff and Wirtz [59] introduced (dioleoyl)phosphatidyl[*N*-*Me*$_3$-^{13}C]choline into the outer monolayer of vesicles of (dimyristoyl)-phosphatidylcholine with a phosphatidylcholine exchange protein. Their ^{13}C NMR measurements indicated that the (dioleoyl)phosphatidylcholine moved to the inner monolayer with a half-time of $<$12 h at 30°C. Therefore, an induction of a change in phospholipid composition of a biological membrane may give a redistribution of molecular species over the two halves of the bilayer. Furthermore, it has been observed that the rate of transbilayer exchange is enhanced in unilamellar vesicles of (dimyristoyl)phosphatidylcholine

in the temperature region of the gel–liquid crystalline phase transition [60].

(*ii*) Glycophorin can be incorporated in lipid vesicles without the use of detergents [61] and the protein spans the lipid bilayer in an asymmetric way with the sugar residues directed to the outside of the vesicles [62]. Van Zoelen et al. [63] reported that [*N*-*Me*$_3$-^{13}C]lysophosphatidylcholine, added to pre-existing glycophorin-containing vesicles is initially incorporated into the outer lipid monolayer; the lysophosphatidylcholine was found to be translocated to the inner monolayer of the glycophorin-containing vesicles with a half-time of about 1.5 h at 4°C [63]. In agreement with these ^{13}C NMR experiments transbilayer movement of lysophosphatidylcholine could also be demonstrated with the action of lysophospholipase on single-bilayer vesicles, prepared by cosonication of lysophosphatidylcholine with phosphatidylcholine. Without the presence of the integral membrane protein only the lysophosphatidylcholine located in the outer monolayer is attacked by lysophospholipase, and no transbilayer movement was observed. Action of lysophospholipase on glycophorin-containing vesicles gave a biphasic hydrolysis pattern resulting in a complete degradation of the lyso-compound. A half-time of translocation of (palmitoyl)lysophosphatidylcholine in glycophorin-containing vesicles of (dioleoyl)phosphatidylcholine was estimated to be about 1 h at 37°C [63].

A transbilayer exchange of phosphatidylcholine itself was also demonstrated in sonicated lipid vesicles containing glycophorin [64]. In a first approach (dioleoyl)phosphatidyl[*N*-*Me*$_3$-^{13}C]choline was introduced into the sonicated vesicles by means of a specific phosphatidyl exchange protein using as donor large, unilamellar glycophorin-containing vesicles consisting of labeled phosphatidylcholine (fig.7). The two types of vesicles are easily separated by centrifugation. The transbilayer distribution of the labeled phosphatidylcholine was measured by ^{13}C NMR (fig.8). In the protein-free acceptor vesicles only a minor quantity of (dioleoyl)phosphatidyl[*N*-*Me*$_3$-^{13}C]choline was found to be present in the inner layer and the fraction did not increase with incubation time. In contrast a substantial quantity of the label appeared to be located at the inner layer of the protein-containing structures demonstrating that glycophorin facilitated the translocation of labeled phospholipid from the outer to the inner layer. Control experiments indicated that under the conditions used the vesicles remain intact barriers and that the exchange protein does not enter the vesicles; it could also be derived that the move-

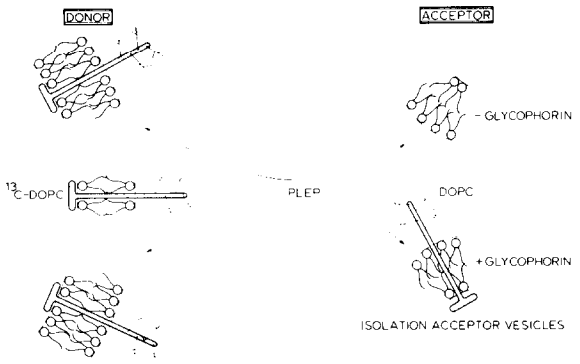

Fig.7. Experimental design for the measurement of transbilayer movement of phosphatidylcholine in sonicated vesicles [64]. Large, unsonicated glycophorin-containing vesicles of (dioleoyl)phosphatidyl [*N*-*Me*$_3$-^{13}C]choline (DOPC) are incubated with sonicated acceptor vesicles, either with or without glycophorin, in the presence of a phosphatidylcholine exchange protein (PLEP). After exchange of about 10% of the phospholipid in the outer layer of the sonicated vesicles for ^{13}C-labelled phospholipid, the acceptor vesicles are separated from the donor vesicles and the exchange protein is removed. The distribution of the label over the outer and inner layer is determined by ^{13}C NMR (fig.8).

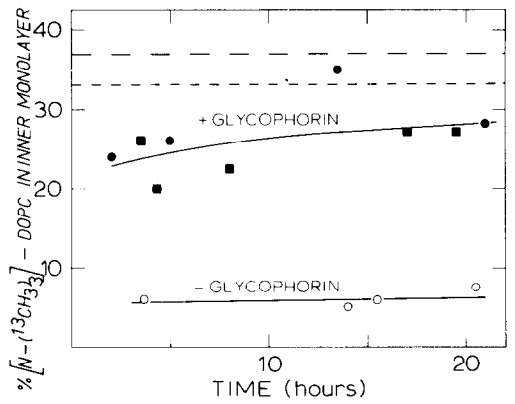

Fig.8. Transbilayer distribution of (dioleoyl)phosphatidyl-[*N*-*Me*$_3$-^{13}C]choline introduced by phosphatidylcholine exchange protein (fig.7) into the outer layer of sonicated vesicles of (dioleoyl)phosphatidylcholine with (●,■) or without (○) glycophorin. ^{13}C NMR measurements were carried out with $DyCl_3$, as shift reagent, after the indicated incubation time, at 27°C. The dashed lines represent the percentage of lipid molecules present in the inner layer of the vesicles with (- -) or without (– –) glycophorin [64].

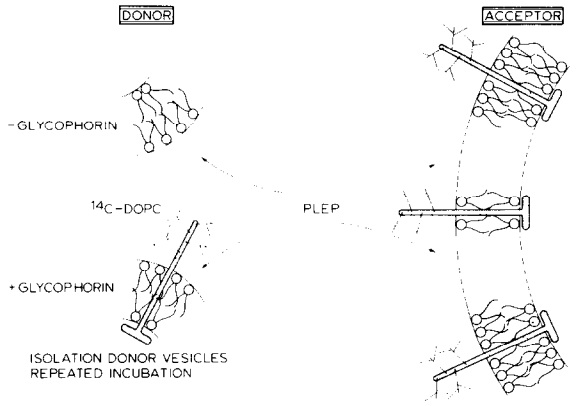

Fig.9. Experimental design for the measurement of transbilayer movement of phosphatidylcholine in sonicated vesicles [64]. Sonicated vesicles of ^{14}C-labelled (dioleoyl)phosphatidylcholine (DOPC), with or without glycophorin are incubated with large, unsonicated glycophorin-containing vesicles in the presence of a phosphatidylcholine exchange protein (PLEP) for 1 h at 37°C; after removal of the unsonicated structures by centrifugation the sonicated vesicles were incubated with a new amount of unsonicated vesicles together with exchange protein and this procedure was repeated several times. After these repetitive incubations the size of the exchanged pool of phosphatidylcholine in the sonicated vesicles was determined (fig.10).

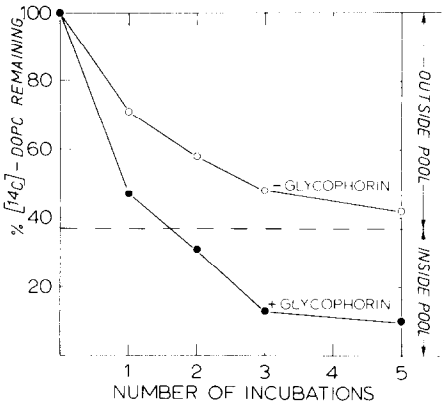

Fig.10. Percentage of (dioleoyl)phosphatidylcholine (DOPC) exchangeable with phosphatidylcholine exchange protein (fig.9) in sonicated vesicles with (•) and without (○) glycophorin. (- -) Percentage of phospholipid molecules present in the inner layer of the sonicated vesicles [64].

ment of phosphatidylcholine from the outer to the inner monolayer is balanced by a translocation in the opposite direction. In the second approach the size of the exchangeable pool of (dioleoyl)phosphatidylcholine in sonicated vesicles was determined by repetitive incubations of the ^{14}C-labelled vesicles with large unilamellar vesicles of unlabeled phosphatidylcholine in the presence of exchange protein (fig.9). The percentage of label that could not be exchanged in the glycophorin-free vesicles approaches the value of the fraction of phospholipid present in the inner layer, confirming that only phospholipid in the outer layer is exchangeable. In glycophorin-containing vesicles at least 90% of the labeled (dioleoyl)phosphatidylcholine was available for exchange, indicating migration of phospholipid from the inner to the outer monolayer (fig.10). Although both approaches do not permit a determination of the exact rate of translocation it is possible to conclude that transbilayer exchange is enhanced by at least two orders of magnitude by the incorporation of glycophorin. Other integral proteins may have a similar effect as was demonstrated by a study with a preparation of partially purified band 3 from erythrocytes [65]. Using the second approach described above, it was found that the band 3-containing vesicles exhibit an exchangeable pool larger than the fraction of phosphatidylcholine in the outer monolayer. Whereas both glycophorin and partially purified band 3 preparations appear to facilitate transbilayer movement, DiCorleto and Zilversmit [66] did not observe an enhanced transbilayer movement of phosphatidylcholine in cytochrome *c* oxidase-containing vesicles. In the latter study the vesicles – having high respiratory control ratio – contained lipid mixtures, whereas in the vesicles with erythrocyte membrane proteins a more simple phospholipid population was utilized. As regards the facilitation of transbilayer exchange of phospholipid by glycophorin it should be noted that its incorporation significantly influences the physical properties of the bilayer [67–71]. ^{31}P NMR studies indicate that glycophorin – by electrostatic interaction – immobilizes a number of lipid molecules very strongly in their phosphate regions (fig.11). Differential scanning calorimetry demonstrated that each glycophorin molecule is able to perturb the properties of 80–100 phospholipid molecules in such a way that these lipid molecules no longer participate in the cooperative gel to liquid–crystalline phase transition, indicating that the perturbation region extends to several layers of phospholipid [69,71]. The presence of glycophorin results in higher susceptibility of the bilayer towards phospholipase action and in enhanced permeability [69]. Therefore, it can be envisaged that the incorporation of this pro-

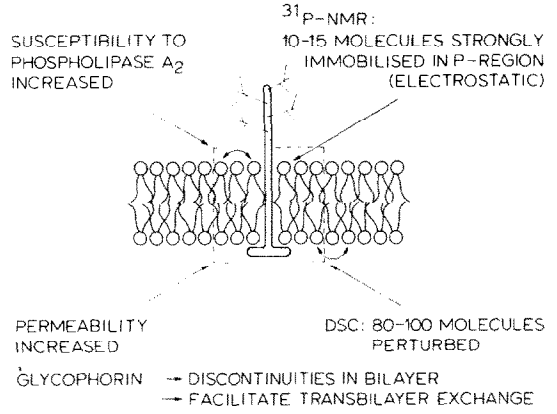

Fig.11. Schematic summary of the effects of the incorporation of glycophorin on the properties of a bilayer of a single phospholipid species [69].

tein in a homogeneous lipid bilayer causes discontinuities in the lipid packing and that such irregularities in the architecture causes regions favourable for transbilayer exchange of phospholipid.

(*iii*) Pioneering X-ray studies of Luzzatti and associates [72–75] already demonstrated at an early stage that in addition to the bilayer arrangement a number of lipid classes can assemble into a variety of quite different phases. In recent years, Cullis, De Kruijff and associates have made detailed studies of lipid polymorphism through the use of ^{31}P NMR, and they have emphasized that such non-bilayer configurations may be involved in a number of dynamic membrane processes including translocation of phospholipids (reviewed in [16]). They proposed a model which involves the transitory formation of inverted micelles in a lipid bilayer which could account for the movement of lipids from one monolayer to the other. This additional mechanism for phospholipid translocation was tested in a model system composed of phosphatidylcholine and cardiolipin. The addition of Ca^{2+} to this system was shown to introduce a structural change involving a non-bilayer phase, indicated by ^{31}P NMR as isotropic motion of the phospholipids [76], and freeze-fracturing made it possible to visualize that Ca^{2+} generates the formation of lipidic particles within the bilayer [77]. Experiments of Gerritsen et al. [78] with a phosphatidylcholine exchange protein showed that in vesicles of phosphatidylcholine–cardiolipin (1:1) without Ca^{2+} the action of the protein is confined to the outer monolayer and that no detectable transbilayer movement of phospholipid occurs. However, the induction of an isotropic phase by Ca^{2+} was accompanied by a considerable increase in phosphatidylcholine exchangeability which indicates that the non-bilayer arrangement causes rapid transbilayer movement of phosphatidylcholine.

The experiments on lipid vesicles have made clear that various factors can induce transbilayer exchange of phospholipids. The future will show whether these results obtained in membrane model systems account for phospholipid flip–flop in biomembranes or whether other, perhaps more specific, translocation mechanisms are involved.

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