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SHORT REVIEW

The Topology of Phospholipids in Artificial and Biological Membranes

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Introduction

Many cellular functions, such as transport processes, transfer of energy, or transmission of signals, are dependent on the molecular architecture of biological membranes and the vectorial orientation of their constituents, i.e., on the asymmetric distribution of the components in the lateral as well as in the transverse plane. This has become clear in recent years for membrane proteins (Steck, 1974; Rothman and Lenard, 1977; De Pierre and Ernster, 1977; Nilsson and Dallner, 1977a; Etemadi, 1980a). On the other hand, the distribution of phospholipids in biological or synthetic membranes is in most cases not absolute, i.e., they can be found in both leaflets of the lipid bilayer.

Since the first reports of Bretscher (1972a,b) about the asymmetric distribution of phospholipids in a biological membrane, an increasing amount of data has accumulated to demonstrate phospholipid asymmetry in many different membranes. Very often the results have been quite controversial, depending on the methods being used. The many discrepancies in this field are due to the many problems inherent in the different techniques to employ. Difference in permeability of various membranes, intactness of the membrane after completion of the reaction, time scale of the latter in relation to possible

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movements of the reactants across the membrane (flip-flop), or accessibility of the components are only a few of the difficulties one might have to deal with.

Several excellent and extensive reviews have appeared in recent years (Rothman and Lenard, 1977; Bergelson and Barsukov, 1977; Op den Kamp, 1979; Etemadi, 1980b; Van Deenen, 1981). The purpose of this review will be twofold: first, to summarize briefly in a rather concise way the available data (which by no means can be complete), and, second, to discuss critically the different techniques which have been applied to unravel the phospholipid distribution in various membranes. I will emphasize the inherent problems of the different methods and will discuss the possible implications of phospholipid asymmetry in membranes. However, no attempts will be made to discuss in detail the origin of phospholipid asymmetry in membrane systems (e.g., see Rothman and Lenard, 1977; Bergelson and Barsukov, 1977).

Chemical Labeling

A number of reagents, originally used to modify amino groups of proteins, have been applied to study the transverse distribution of aminophospholipids in different membranes, mainly phosphatidylethanolamine and phosphatidylserine. In 1972 Bretscher (1972a,b) first demonstrated that in intact erythrocytes phosphatidylethanolamine and phosphatidylserine were not labeled by the relatively impermeant reagent ^{35}S-Formylmethionyl(sulfone)methylphosphate, whereas in unsealed ghosts both aminophospholipids became modified. From these results he concluded that an asymmetric distribution of the phospholipids had to exist across the erythrocyte membrane. Shortly later, these results were confirmed by Gordesky and Marinetti (1973). By using the nonpermeant label 2,4,6-trinitrobenzenesulfonate (TNBS) they reported that about 20% of phosphatidylethanolamine, but no phosphatidylserine, had been labeled in intact cells. On the other hand, in unsealed ghosts the aminophospholipids could be labeled almost completely.

Initially, the concept of an asymmetric distribution of phospholipids in biological membranes was criticized, since Bretscher based his conclusions on initial rate measurements. By using such an approach, the different reactivities of the lipids on either side of the membrane could not be excluded from consideration. Furthermore, it was argued that the properties of the membrane were changed due to the formation of ghosts. Whiteley and Berg (1974) eliminated this criticism. They used analogous reagents, one of which could easily penetrate the membrane (ethylacetimidate, EAI), whereas the other (isethionylacetimidate, IAI) could not. They were able to show that intact cells were labeled to the same extent by EAI as unsealed ghosts. On the other

hand, in ghosts aminophospholipids were modified to a much greater extent by IAI than in intact cells.

Later, the nonpenetrating reagent TNBS became widely used together with the permeating probe 1-fluoro-2,4-dinitrobenzene (FDNB) in order to determine the distribution of aminophospholipids across various membranes. These studies included plasma membranes of different origins such as erythrocytes (Van Deenen, 1981; Gordesky *et al.,* 1975; Marinetti and Love, 1976; Haest and Deuticke, 1975; Haest *et al.*, 1981), platelets (Schick *et al.*, 1976), fibroblasts (Mark-Malchoff *et al.,* 1977; Sandra and Pagano, 1978; Fontaine and Schroeder, 1979), synaptosomes (Smith and Loh, 1976; Fontaine *et al.*, 1979, 1980) and bacteria (Gordesky *et al.*, 1975; Marinetti and Love, 1976; Rothman and Kennedy, 1977a,b; Bishop *et al.,* 1977; Shimada and Murata, 1976; Paton *et al.*, 1978; Demant *et al.*, 1979; Kumar *et al.*, 1979), membranes from various subcellular organelles such as mitochondria (Marinetti *et al.,* 1976; Crain and Marinetti, 1979; Cheesbrough and Moore, 1980), sarcoplasmic reticulum (Vale, 1977), retinal rod outer segments (Smith *et al.,* 1977; Crain *et al.,* 1978; Drenthe *et al.*, 1980) and glyoxysomes (Cheesbrough and Moore, 1980), membrane envelopes of some viruses (Fong *et al.*, 1976; Fong and Brown, 1978) and liposomes (Litman, 1973; Lentz and Litman, 1978; Lee and Forte, 1979).

In determining the phospholipid distribution by a variety of different methods, several basic points must be considered:

(1) Permeation of the reagent through the membrane
(2) Intactness of the membrane after completion of the reaction
(3) Accessibility of the substrate
(4) Time course of the reaction
(5) Homogeneous polarity of the membrane

As mentioned before, TNBS is considered to be a nonpenetrating reagent. However, it has been shown that under certain conditions even charged reagents like TNBS can pass through the phospholipid bilayer and react with aminophospholipids on both sides of the membrane. Thus it was reported by Vale (1977) that in vesicles of sarcoplasmic reticulum, labeling of phosphatidylethanolamine reaches a plateau at certain concentrations of TNBS. This value corresponds to 70% of the total phosphatidylethanolamine. If the concentration of TNBS was raised further, additional labeling could be observed. This result was interpreted as an indication that higher concentrations of the label facilitate the penetration of the reagent through the membrane. Marinetti and co-workers (1976) presented evidence that erythrocytes were not permeable to TNBS. Under the assumption that this is also true for the inner mitochondrial membrane, Marinetti *et al.* (1976) reported that

about 60% of the total phosphatidylethanolamine of the inner membrane of rat liver mitochondria was located on the cytoplasmic side. In a later publication, Crain and Marinetti (1979) showed that TNBS indeed can penetrate the inner mitochondrial membrane. Using a detailed kinetic analysis, they concluded that only about 40% of phosphatidylethanolamine was located on the cytoplasmic side. Also Rothman and Kennedy (1977a) found TNBS to be able to penetrate the membrane of *B. megaterium,* provided the membranes were incubated at 15°C. This was in contrast to results obtained at 0°C.

Recently, Haest *et al.* (1981) showed that erythrocytes can become permeable to TNBS. They noticed that the high glutathione content of human erythrocytes rapidly diminished if the cells were incubated with TNBS. Therefore, hemoglobin was labeled only if the reaction of TNBS with glutathione was completed. Since inhibitors of the anion-transport system prevented the uptake of TNBS by the erythrocytes, the authors concluded that the label enters the cells by using this pathway. These results underline the importance of evaluating the labeling conditions for each kind of membrane separately. It cannot be assumed that the permeability barrier for a reagent is similar or even the same in different membranes.

It has often been pointed out that chemical labeling, even if restricted in most cases to aminophospholipids, has its merits, since most of these probes are of small size. Therefore they should be able to react with phospholipids which are otherwise inaccessible. Nevertheless, incomplete labeling has been reported for different membrane systems (Gordesky and Marinetti, 1973; Marinetti and Love, 1976; Bishop *et al.,* 1977; Crain and Marinetti, 1979; Vale, 1977). This observation can be explained by the introduction of negatively charged groups at the surface of the membrane which can cause restrictions by preventing the reaction from going to completion.

A further important point which has to be considered before choosing a label is the time course of the reaction. It is obvious that reagents which complete their reaction immediately, also at low temperatures, have several advantages, especially considering the possibility of transbilayer movement of phospholipids. Such a process has been reported to occur in some biological membranes (Van Deenen, 1981) and will be discussed in detail later. One reagent bearing these properties is fluorescamine. The reaction of this probe, developed by Udenfriend and co-workers (1972), usually is completed within less than a second, even at 4°C. This reagent has been used to study the transverse localization of phosphatidylethanolamine and phosphatidylserine in sarcoplasmic reticulum. Hasselbach and co-workers (Hasselbach *et al.,* 1975; Hasselbach and Migala, 1975), by using fluorescamine, as well as Hidalgo and Ikemoto (1977), by applying the fluorescamine–cycloheptaamylose complex (Nakaya *et al.,* 1975), reported that 70–80% of phosphatidyle-

thanolamine was located on the cytoplasmic side, whereas phosphatidylserine was not accessible to the reagents. These results were comparable to the figures published by Vale (1977) using TNBS as the probe.

Fluorescamine was also successfully applied to investigate the distribution of phosphatidylethanolamine in the inner membrane of beef heart mitochondria (Krebs *et al.*, 1979). Membranes of opposite polarity have been used. The homogeneity of these preparations with respect to the orientation of their constituents was demonstrated to be better than 90%; 40% and 60% of the total phosphatidylethanolamine could be labeled in mitoplasts (= right-side out oriented membranes) and submitochondrial particles (= inside out oriented vesicles), respectively, under nonpenetrating conditions for the label. At higher concentrations of the label, the amount of modified phosphatidylethanolamine increased further until about 95% of the total phosphatidylethanolamine had reacted, indicating the penetration of the probe through the membrane. In addition, the fluorescamine–cycloheptaamylose complex, which due to its higher molecular weight should be less permeable (Hidalgo and Ikemoto, 1977), has been applied. Using this complex, saturation values have been approached for mitoplasts at 38% and for submitochondrial particles at 63% of the total phosphatidylethanolamine. Similar results were recently obtained for rat liver mitochondria (Toni and Krebs, unpublished), confirming the results reported by Crain and Marinetti (1979).

Lee and Forte (1979) used fluorescamine and TNBS to label asymmetrically phosphatidylethanolamine in mixed phosphatidylcholine/phosphatidylethanolamine liposomes. They reported that an effective concentration of 40% of phosphatidylethanolamine was located in the outer monolayer. This value is in excellent agreement with figures reported by Litman (1973) using TNBS as a probe. It also agrees with the results obtained by using nuclear magnetic resonance methods (Berden *et al.*, 1975; Krebs and Hauser unpublished).

Immunological Methods

Most lipids showing an immunological reaction are either neutral lipids or glycolipids. However, acidic phospholipids such as cardiolipin, phosphatidylglycerol or phosphatidylinositol display serological properties (Rapport and Graf, 1969) in contrast to the zwitterionic phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine) with the exception of sphingomyelin (Teitelbaum *et al.*, 1973). The antigenic determinant is usually constituted by the polar head group. Nanni *et al.* (1969) and later Guarnieri *et al.* (1971) were the first to attempt the determination of phospholipids in different membranes by applying immunological methods. Nanni *et al.* (1969)

obtained antibodies against the phospholipids of erythrocytes from sheep. They used the antigen–antibody reaction to localize sphingomyelin mainly at the outer surface of the membrane, whereas phosphatidylethanolamine and phosphatidylserine were placed mainly at the cytoplasmic side. These rather preliminary results were nevertheless confirmed later by more detailed and quantitative studies by a number of other laboratories (Gordesky and Marinetti, 1973; Verkleij *et al.*, 1973; Crain and Zilversmit, 1980; Van Meer *et al.*, 1980a). On the other hand, Guarnieri *et al.* (1971) studied the localization of cardiolipin in mitochondria from different origins, but were unable to detect a reaction between the anticardiolipin antibodies and cardiolipin in the inner membrane of mitochondria to an appreciable extent. Even inside-out submitochondrial particles from beef heart mitochondria, depleted of the F_1-ATPase, did not show any noticeable reaction. Therefore they concluded that most of the cardiolipin was not available for the reaction with the antibodies. But since the detection methods used by these authors were rather insensitive, their interpretation was misleading. Indeed, Schiefer (1973a,b) in two later publications showed that interactions between anticardiolipin antibodies and the inner mitochondrial membrane (Schiefer, 1973a,b) and phosphatidylinositol-antibodies and microsomal membranes (Schiefer, 1973b) did occur. But although he was able to detect even small accessible amounts of the phospholipid in the respective membrane, especially after pronase treatment of the membranes, Schiefer could not quantitate his results. In a later attempt a quantitative estimate of the cardiolipin distribution in the inner membrane of beef heart mitochondria was made (Krebs *et al.*, 1979). Trypsinization of these membranes also exposed additional antigenic sides, especially in the inside-out submitochondrial vesicles. The results indicated that cardiolipin was three times enriched on the matrix side of the inner membrane of beef heart mitochondria (Krebs *et al.*, 1979) as compared to the cytoplasmic side.

Several other studies have been undertaken to use the serological properties of some phospholipids for the determination of their transverse membrane distribution, but only qualitative results could be obtained (Radunz, 1971; Schiefer *et al.*, 1975; Guarnieri, 1975). Interestingly, also in two of these reports proteolytic digestion revealed more antigenic sites for the antibody reaction (Radunz, 1971; Schiefer *et al.*, 1975), indicating that a substantial part of the phospholipids was masked by proteins.

Phospholipase Digestion of Membrane Phospholipids

Enzymatic degradation of phospholipids is a widely used technique to determine the transmembrane distribution of various phospholipids. Several

classes of phospholipases from a number of different sources have been used, mostly phospholipase A_2, *C*, *D* and sphingomyelinase (Roelofsen and Zwaal, 1976). Since phospholipases are thought not to penetrate intact biological membranes, they should provide ideal tools to attack phospholipids on the outer surface of a membrane. But several factors have to be borne in mind:

(1) Substrate specificity
(2) Membrane surface pressure
(3) Lysis of the membrane

Phospholipase A_2 from different sources reveals remarkable substrate specificities. Thus it has been shown that acidic phospholipids, especially cardiolipin, serve as poor substrates (Marinetti, 1964; Okuyama and Nojima, 1965; Krebs *et al.*, 1979). Similar observations have been reported for phospholipase *C* from *Clostridium welchii* (Möllby and Wadström, 1973). Furthermore, it is interesting to note that phospholipase A_2 from *N. naja* venom can show a reversal of substrate specificity depending on whether the phospholipids have been assayed individually or as a mixture of phospholipids (Adamich and Dennis, 1978a,b). A further aspect to be considered is the fact that in venoms from different sources phospholipase A_2 often exists as a mixture of isozymes, showing different substrate specificity and lytic properties (Salach *et al.*, 1968; Lankisch *et al.*, 1971; Martin *et al.*, 1975). This might be one reason for the controversy concerning the applicability of snake venom phospholipase A_2 in phospholipid distribution studies (Higgins and Dawson, 1977; Nilsson and Dallner, 1977b; Sundler *et al.*, 1977; see also Krebs *et al.*, 1979; Martin *et al.*, 1975).

Another important factor which might play some role in the application of phospholipases is the surface pressure of a membrane. It has been observed that pancreatic phospholipase A_2 as well as phospholipase *C* from *B. cereus* do not degrade any phospholipids in intact human erythrocytes. This is in contrast to the results obtained by using phospholipase A_2 from *N. naja* which can hydrolyze 68% of the total phosphatidylcholine without lysing the cell (Verkléij *et al.*, 1973; Zwaal *et al.*, 1975). Treatment of the cells with pronase prior to the incubation with phospholipases did not influence the results (Roelofsen *et al.*, 1971), so that a shielding effect by proteins can be excluded. The difference in the reactivity of the various phospholipases versus the intact erythrocytes could be interpreted thus: the surface pressure of the intact cell might be too high to enable phosphopholipase *C* from *B. cereus* as well as the pancreatic phospholipase A_2 to attack phospholipids at the surface of the membrane. This view is supported by the fact that in monolayer studies with various initial surface pressures, it has been observed that pancreatic phospholipase A_2 and phospholipase *C* from *B. cereus* can hydrolyze phospholipids

only at initial surface pressures below 30 dynes/cm whereas phospholipase A_2 from *N. naja* can react at values higher than 30 dynes/cm (Zwaal *et al.*, 1975; Zwaal, 1974).

Nevertheless, a combination of the different phospholipases hydrolyzing the phospholipids under nonlytic conditions in intact cells as well as digesting the lipids in unsealed ghosts led to a consistent picture of the phospholipid distribution in the erythrocyte cell. More than 80% of sphingomyelin, 75% of phosphatidylcholine, and 20% of phosphatidylethanolamine were located at the exterior surface, whereas at the cytoplasmic side mainly the aminophospholipids, especially phosphatidylserine, were concentrated (Verkleij *et al.*, 1973). These data were supported by experiments in which phospholipases were applied from the inside of resealed ghosts (Zwaal *et al.*, 1975).

The results obtained were in full agreement with the more indirect data obtained by Bretscher (1972a,b) and Gordesky and Marinetti (1973) by chemical labeling. They were also supported by experiments using phospholipid-exchange proteins, as will be discussed below.

Phospholipid Exchange Proteins

The presence of phospholipid exchange proteins can enhance considerably the spontaneous exchange of phospholipids between different membranes. This was first demonstrated by Wirtz and Zilversmit (1968). A variety of different phospholipid-transferring proteins have been isolated. They were either highly specific for one class of phospholipids, like phosphatidylcholine (Kamp *et al.*, 1973, 1977), or nonspecific, translocating different phospholipids from one membrane to the other (e.g., Zilversmit and Hughes, 1977; Crain and Zilversmit, 1980; for reviews see Wirtz, 1974; Zilversmit and Hughes, 1976). Since these proteins cannot penetrate the membrane, they accept phospholipids only from the outer monolayer of the membrane. This has been shown for different donor membranes such as erythrocytes (Crain and Zilversmit, 1980; Bloj and Zilversmit, 1976; Van Meer *et al.*, 1980a), bacteria (Barsukov *et al.*, 1976), and liposomes (Johnson *et al.*, 1975; Rothman and Dawidowicz, 1975; Low and Zilversmit, 1980).

It is further assumed that such an exchange of phospholipids does not perturb the structure of the membrane. Therefore exchange proteins can provide very useful tools to characterize the transbilayer distribution of the phospholipid constituents of a membrane, provided the latter are freely accessible to the proteins (Van Meer *et al.*, 1980a). By using a phosphatidylcholine-specific exchange protein (Van Meer *et al.*, 1980a) or a nonspecific exchange protein (Crain and Zilversmit, 1980), the phospholipid distribution data described before for the erythrocyte membrane were confirmed. In both

reports it was demonstrated that more than 70% of the choline-containing phospholipids were concentrated in an easily accessible pool whereas the concentration of phosphatidylethanolamine in the outer layer was rather low.

In addition, phospholipid exchange proteins are ideal tools to follow transbilayer movement of phospholipids. So it became obvious from experiments with phosphatidylcholine vesicles that about 60% of the total amount of the lipid, which corresponds to the phospholipids being in the outer shell of the vesicle, was readily available. On the other hand, about 40% of the phospholipids became accessible at a much slower rate, indicating that the latter value corresponds to the phospholipids located in the inner layer of the vesicles, slowly moving across the bilayer (Johnson *et al.*, 1975; Rothman and Dawidowicz, 1975). Similar observations of pools with a different rate of transfer have also been reported for erythrocytes (Crain and Zilversmit, 1980; Bloj and Zilversmit, 1976). In contrast, microsomes apparently consist only of one pool of phospholipids which is readily available for the different exchange proteins (Zilversmit and Hughes, 1977; van den Besselaar *et al.*, 1978; Jackson *et al.*, 1978; Brophy *et al.*, 1978). This result has been interpreted as indicating a rapid transbilayer movement of the various phospholipids in the microsomal membrane. This observation and its implications will be discussed in detail below together with results obtained by using nuclear magnetic resonance techniques.

Physicochemical Methods

Various spectroscopic, magnetic resonance, or X-ray diffraction techniques have been applied in order to obtain detailed information about the structure and dynamics of a membrane on the molecular level (for a review, see *Membrane Spectroscopy,* 1981). In particular, nuclear magnetic resonance (NMR) has been successfully applied to establish the distribution of lipids in small, sonicated vesicles of various phospholipid mixtures. Bergelson and co-workers (1971) introduced the use of paramagnetic ions which do not penetrate the bilayer and either shift or broaden the signal from those lipids which are in close contact with the reagents. Using this technique, they were able to differentiate between the phospholipids located in the inner and outer monolayer. Under suitable conditions the data can be quantitated (Bergelson and Barsukov, 1977). In a detailed study Berden *et al.*, (1975) determined the phospholipid distribution in mixed lipid vesicles. The authors were able to show that acidic phospholipids were preferentially located in the inner monolayer of the vesicles whereas choline containing phospholipids showed a preference for the outer leaflet. In the case of phosphatidylcholine/phosphatidylserine mixtures, a change of pH can influence the phospholipid distribution

in those vesicles. At low pH-values phosphatidylserine was preferentially found on the inner side, whereas at alkaline pH values phosphatidylserine was mainly found in the outer monolayer of the vesicles. From their data the authors concluded that the ratio between the surface area occupied by the polar head group and the charge density at the polar head group can strongly influence the distribution of the phospholipids across the bilayer. Similar results have been reported recently by Barsukov *et al.* (1980) using a slightly different approach.

Even if NMR techniques can provide useful information on phospholipid distribution in membranes, the technique (especially ^{31}P-NMR) has been successfully applied only to single-shelled lipid vesicles of small diameter (300–500 Å). This is due to the isotropic tumbling of these vesicles and the fast lateral diffusion of lipid molecules which can average out the effects of chemical shift anisotropy and dipole–dipole interactions on the signal (Finer *et al.*, 1972; Bloom *et al.*, 1975). In larger vesicles or biological membranes, chemical shielding anisotropy becomes a dominating factor which can prevent a quantitative estimate of the phospholipid transverse distribution.

On the other hand, chemical shift anisotropy of the phosphorus signal can provide useful information about possible phase changes of lipids within the membrane (for a review see Cullis and de Kruijff, 1979, and Seelig, 1978). Since the classical work of Luzzati and his co-workers (1962, 1966, 1968), it has been known that certain phospholipids in the hydrated state, e.g., phosphatidylethanolamine (Reiss-Husson, 1967; Rand *et al.*, 1971) or cardiolipin (Rand and Sengupta, 1972), can adopt different phases (i.e. lamellar, hexagonal, or inverted micellar, cubic, or rhombic phases), depending on the experimental conditions. These phases are reflected by the line shape of the ^{31}P-NMR spectrum.

In a series of papers Cullis, de Kruijff, and co-workers (1979) studied the lipid polymorphism in detail, in artificial as well as in biological membrane systems, by using ^{31}P-NMR techniques. From their results they concluded that membranes with a high content of choline-containing phospholipids were stabilized in the bilayer lamellar phase. They were able to show that erythrocytes retained their lamellar phase, even after extensive digestion of the phospholipids by phospholipases (Van Meer *et al.*, 1980b), thereby producing lipids which could induce nonbilayer structures. Lipids undergoing isotropic motion have been claimed to occur in intracellular membranes like those of the endoplasmatic (Stier *et al.*, 1978; de Kruijff *et al.*, 1978) or sarcoplasmic reticulum (de Kruijff *et al.*, 1979), as supported by ^{31}P–NMR spectra.

The reason for isotropic motion of part of the phospholipids within the membrane could be an inverted micellar arrangement of the lipids which could eventually facilitate their fast transbilayer movement. This view was

supported by results obtained by using phospholipid exchange proteins (Zilversmit and Hughes, 1977; van den Besselaar *et al.*, 1978; Jackson *et al.*, 1978; Brophy *et al.*, 1978), suggesting one pool of phospholipids available for exchange as mentioned before. Furthermore, this could also explain the rather controversial reports of the phospholipid distribution in microsomal membranes by three different laboratories (Higgins and Dawson, 1977; Nilsson and Dallner, 1977b; Sundler *et al.*, 1977) as mentioned before. But the reason for the isotropic motion of phospholipids within a membrane might also be the tumbling of vesicles with a small diameter or the fast lateral diffusion of the lipids on the surface of highly curved membranes. Those factors can, in particular, play a role in microsomal membrane preparations. In this respect it is interesting to note that Fleischer *et al.* (1979) reported NMR data obtained from sarcoplasmic reticulum vesicles with greater than 90% of the phospholipids in the lamellar bilayer phase.

Inner mitochondrial membranes have a high content of phosphatidylethanolamine and cardiolipin. These phospholipids can undergo a lamellar/hexagonal phase transition in model membrane systems, as shown by X-ray diffraction (Reiss-Husson, 1967; Rand *et al.*, 1971; Rand and Sengupta, 1972) as well as by ^{31}P-NMR techniques (Cullis and de Kruijff, 1978; Cullis *et al.*, 1978). Therefore it is quite interesting that apparently in the inner mitochondrial membrane these lipids are stabilized in the lamellar bilayer configuration, even at 37°C, as demonstrated by ^{31}P-NMR (Cullis *et al.*, 1980). This might be the reason for the very slow transbilayer movement of phospholipids in this membrane ($t_{1/2} > 24$ h) as shown by electron paramagnetic resonance experiments (Rousselet *et al.*, 1976). These data might also explain the finding of an asymmetric distribution of phospholipids across this membrane (Crain and Marinetti, 1979; Krebs *et al.*, 1979).

Conclusions

To date the most conclusive evidence for the asymmetric distribution of phospholipids across a plasma membrane—if not any biological membrane—has been obtained for the red blood cell membrane. Several laboratories using a variety of different techniques as described above obtained consistent results. It has been shown that choline-containing phospholipids are preferentially located in the outer layer whereas aminophospholipids are found mainly on the cytoplasmic side (Bretscher, 1972b; Gordesky and Marinetti, 1973; Verkleij *et al.*, 1973; Zwaal *et al.*, 1975; Crain and Zilversmit, 1980; van Meer *et al.*, 1980a). A similar distribution has also been described for the plasma membrane of platelets (Chap *et al.*, 1977). In the context of these results, an interesting argument for the physiological significance of phospholipid asym-

metry in red blood cells has been put forward by Zwaal *et al.* (1977). Their experiments indicated that phosphatidylethanolamine and phosphatidylserine, the lipids of the inner monolayer of blood cell membranes, could be involved in the regulation of the blood coagulation process, in contrast to the choline-containing lipids present mainly in the outer layer. The asymmetric distribution of the phospholipids across the erythrocyte membrane may be stabilized by membrane proteins, since experiments from different laboratories indicated that spectrin, a membrane protein located at the cytoplasmic side, can stabilize aminophospholipids at the surface of the inner layer of the membrane (Haest *et al.,* 1978; Marinetti and Crain, 1978; Mombers *et al.,* 1980). Furthermore, it might also be of interest that the Ca^{2+}-ATPase of erythrocytes, a membrane protein with the active center facing the cytoplasmic side, can be stimulated quite considerably by acidic phospholipids (e.g., phosphatidylserine), as shown by Niggli *et al.* (1981).

The phospholipid distribution of other plasma membranes has also been studied using a rather indirect technique. Based on the assumption that the budding out process of a virus should envelop the viral body with a lipid bilayer, reflecting the orientation of the host cell membrane, different viral membranes have been investigated. Two studies with different viruses described a lipid distribution comparable to that found in erythrocytes (Fong *et al.,* 1976; Tsai and Lenard, 1975; Lenard and Rothman, 1976).

In another study using the same virus and the same host cell, completely different observations have been made (Rothman *et al.,* 1976). According to that report sphingomyelin was mainly located at the inner side of the membrane, whereas phosphatidylethanolamine and phosphatidylserine were equally distributed across the membrane. These controversial results may indicate that the viral membrane might not directly reflect the membrane of the host cell.

As outlined before, the existence of an asymmetric distribution of phospholipids in membranes of several intracellular organelles is, at least in some cases, still a matter of debate. On the one hand, the phospholipid asymmetry was well demonstrated in the inner membrane of mitochondria from different sources by two different groups using a variety of different techniques (Crain and Marinetti, 1979; Krebs *et al.,* 1979). These results indicate the preferential location of phosphatidylcholine at the cytoplasmic side and of phosphatidylethanolamine and cardiolipin at the matrix side. On the other hand, an asymmetric phospholipid distribution was also demonstrated for mitochondria from plant cells (Cheesbrough and Moore, 1980), but the somewhat different results were difficult to compare with those described above, since in the latter study 40% of the total phospholipids were not accessible to the reagents.

It should be pointed out that mitochondrial phospholipids with a high

content of unsaturated fatty acids (phosphatidylethanolamine and cardiolipin) or with a negatively charged polar head group (cardiolipin) are apparently concentrated on the matrix side of the inner mitochondrial membrane. Since the protein/phospholipid ratio of this membrane is rather high, with a considerable amount of proteins being located on the matrix side (De Pierre and Ernster, 1977), the environment of phospholipids with a high content of unsaturated fatty acids may have an important influence on the function of mitochondrial membrane proteins. Thus it has been indicated that cardiolipin may participate in the function of mitochondrial membrane enzymes like the cytochrome *c* oxidase (Vik *et al.*, 1981) or the mitochondrial ATPase (Ernster *et al.*, 1977). The phospholipid distribution across the membrane might also have some impact on the rotational mobility of mitochondrial membrane proteins (Kawato *et al.*, 1980, 1981).

The phospholipid distribution in microsomal membranes is much less clear. While two groups report an asymmetric distribution of opposite polarity (Higgins and Dawson, 1977; Nilsson and Dallner, 1977b), Sundler *et al.* (1977) came to the conclusion that the phospholipids were evenly distributed across the membrane. This rather controversial situation might be due to the inherent problems of the applied techniques, as outlined before, or it might reflect the high phospholipid turnover of these membranes, which could result in a rather fast flip-flop movement of the phospholipids across the membrane (Zilversmit and Hughes, 1977; van den Besselaar *et al.*, 1978; Jackson *et al.*, 1978; Brophy *et al.*, 1978; see also Stier *et al.*, 1978; de Kruijff *et al.*, 1978).

The data available from studies of bacterial membranes also do not provide a clear picture of the phospholipid distribution across these membranes (Rothman and Kennedy, 1977a,b; Bishop *et al.*, 1977; Shimada and Murata, 1976; Paton *et al.*, 1978; Demant *et al.*, 1979; Kumar *et al.*, 1979). But since the phospholipid composition of these membranes can easily be altered due to the different cultivating conditions, this might not be too surprising. An interesting observation has been reported by Rothman and Kennedy (1977a). They provided evidence that in *B. megaterium* phosphatidylethanolamine was preferentially located at the inner monolayer of the cytoplasmic bacterial membrane. The asymmetric distribution of the phospholipid across the membrane was not disturbed by phospholipid biosynthesis. Newly synthesized phosphatidylethanolamine, which first occurs at the cytoplasmic side of the membrane, could be found shortly after in the exterior layer of the membrane (Rothman and Kennedy, 1977b). This indicates that certain factors, eventually specific phospholipid–protein interactions, might maintain the transmembrane distribution of phospholipids, even if phospholipid biosynthesis could unbalance it and fast transbilayer movement of the phospholipids apparently occurs.

These data shed some light on the possibility that even if the asymmetric

distribution of phospholipids across a membrane is a somewhat static picture, its maintainance might still have important implications for a variety of membrane processes. Such a phospholipid asymmetry could introduce differences in fluidity into the two monolayers of a membrane or a charge separation, the latter due to the asymmetric distribution of acidic phospholipids.

It has also been argued that regions of high curvature, which can influence differently the surface tension of the inner and outer monolayer of a membrane, might depend on the phospholipid asymmetry. Clearly, further studies are needed in order to gain a more conclusive picture of the topology of phospholipids in the various membranes as well as of its biological significance.

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