Topological Asymmetry of Phospholipids in Membranes

The distribution of phospholipids in biological membranes is related to that in bilayer membranes of small vesicles.

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Many data have implied that cell membranes are composed basically of a lipid bilayer with interdispersed proteins. The polar groups of the lipids are exposed at the surfaces and the fatty acid chains occupy the interior. The lipid bilayer usually contains a wide variety of chemically distinct phospholipids, its actual composition being determined, at least in part, by the functional requirements of the membrane. In order to clarify the role of lipids in membrane function we must have a complete picture of the topological distribution of various lipid species within the membrane. To fulfill its vital functions the membrane must operate differentially on the two compartments it separates, and thus it must be asymmetric, that is, its outer surface must differ chemically from the inner one. The asymmetrical placement of the proteins within the lipid matrix has been studied extensively in many membranes, but much less is known about the asymmetry of the lipid bilayer itself.

In an aqueous environment phospholipids spontaneously form bilayers of the same structure as that providing the framework of biological membranes. The study of such artificial phospholipid bilayers has been of great help in understanding the structure of natural membranes. Swelling of phospholipids in water may result in the formation of spherical structures composed of concentric bilayers with water trapped between the lamellae (coarse liposomes). When the coarse liposomes are exposed to ultrasound they are broken down and reseal into closed, fairly homogeneous small vesicles of about 250 Å (outer diameter). The liposomes thus formed are comprised of an aqueous space surrounded by a single bilayer which is about 50 Å thick (*1*). In sonicated liposomes composed of mixtures of different phospholipids the phospholipids distribute asymmetrically between the outer and the inner shells of the bilayer. The driving forces of this asymmetry are of a thermodynamic nature, depending mainly on steric and electrostatic interactions between the different phospholipids. The viewpoint we develop in this article is that the asymmetric distribution of phospholipids in biological membranes may be related to the trans-bilayer orientation of lipids observed in artificial, highly curved vesicles.

Experiments designed for studying the composition of the outer surface of a membrane are usually based on the interaction of intact cells or sealed vesicles with agents (probes) that are thought not to penetrate the membrane barrier. The composition of the inner surface may then be evaluated from the interaction of the same probe with lysed cells or disrupted membrane preparations in which both surfaces are accessible, or with inside-out oriented vesicles that frequently are formed by the resealing of disrupted membranes. Alternatively, the results of experiments with nonpermeating probes may be compared to those obtained with readily penetrating agents. Nonreactive membrane components shielded by proteins or carbohydrates can be made accessible to the probe by limited proteolysis or by glycosidase treatment.

The results of such studies are valid only if the following conditions are satisfied: (i) the asymmetry of the membrane under investigation persists during the experiment; (ii) the structure of the disrupted or resealed membrane preparation is the same as that of the native membrane; (iii) vesicles of right-side-out or inside-out orientation and disrupted nonvesicular membranes can be prepared in a sufficiently pure form with minimal cross contamination; and (iv) when penetrating as opposed to nonpenetrating probes are used, the reactions are comparable on both sides of the membrane.

Actually, none of these conditions is ever fulfilled completely. (i) Transmembrane tumbling of phospholipids (flip-flop), being a thermodynamically unfavorable event, occurs in liposomes only slowly with a half-time of at least several hours (*2–4*). However, data on flip-flop rates across native membranes are contradictory. For erythrocyte membranes some authors have found a flip-flop half-time of about 20 to 30 minutes (*5*), while others have reported half-times of 2.3 hours for ghost membranes (*6*) and of more than 24 hours for intact erythrocytes (*7*). Flip-flop rates may depend critically on the conditions of membrane preparation and in some model systems are enhanced by small amounts of lysolecithin (*8*). (ii) During the preparation of membrane ghosts, structural changes may occur, although the ghosts appear to be sealed and selective permeability is restored. The magnitude of these changes is a subject of controversy (*6, 9–12*). (iii) Membrane preparations are usually not homogeneous because of incomplete rupture or resealing of disrupted membranes. The exact amount of such "impurities" is difficult to evaluate. (iv) The acidity, osmolarity, and ion composition outside and inside a cell or vesicle are usually quite different. Whereas the conditions outside a cell can be changed or held constant as desired, inside conditions are not so readily controlled.

Despite such difficulties membrane sidedness can be mapped if the complicating factors are taken into account and several independent methods are used.

The methods available for studying transversal phospholipid distribution in artificial and biological membranes fall into five general categories: chemical labeling; nuclear magnetic resonance (NMR) spectroscopy with shift and broadening reagents; enzymatic splitting by phospholipase; protein-mediated phospholipid exchange; and immunochemical methods.

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Chemical Labeling

Small electrophylic molecules capable of combining covalently with amino groups have been used for many years to label surface proteins [for a review see (*13*)]. Recently, such molecules have also been used for the labeling of phospholipids containing amino groups, mainly phosphatidylethanolamine and phosphatidylserine.

Reagents used for the labeling of aminophospholipids include readily penetrating probes (*11–20*) such as 1-fluoro-2,4-dinitrobenzene and acetic anhydride, as well as slowly or nonpenetrating substances, mostly compounds carrying sulfo groups (*15–19, 21–26*). A versatile reagent of the last type is the diazonium salt of sulfanilic acid (*25*), which modifies not only phospholipids containing amino groups but also phosphatidylglycerol. Other methods claimed as useful for the labeling of phospholipids on the outer cell surface include treatment with fluorescamine (*27*) or with CNBr-activated dextran (*28*), and lactoperoxidase-catalyzed iodination (*29*).

Although most of these labeling methods are restricted to lipids carrying amino groups, and such lipids frequently comprise only a minor part of the total membrane phospholipids, chemical labeling has some important merits. Its main advantage is that the low molecular weight chemical probes, because of their small size, are able to label phospholipid molecules which are sterically unavailable to phospholipases or other proteins. The reaction products can easily be detected by spectrophotometry or by using radioactive probes. However, the results of chemical labeling experiments may be misleading because the reagents as well as the modified phospholipids formed may cause perturbations of the membrane structure. Sometimes the induced alterations manifest themselves in lysis of the cells or vesicles. In erythrocytes, for example, the aminoreacting probes have been shown to induce measurable cation leak and hemolysis (*15, 30*). In other cases the perturbations may be more subtle and difficult to detect.

Another shortcoming of chemical labeling comes from the fact that the acidity of the outer medium and the cytoplasm is usually different. Since the reactivity of amino groups depends strongly on the *p*H of the medium, such differences may be sufficient to influence the rate and extent to which outward and inward facing aminolipids can react with the probe. The *p*H difference may even increase as the reaction proceeds, making the results obtained with penetrating and nonpenetrating agents difficult to compare (*9*).

NMR Spectroscopy with Shift and Broadening Reagents

Much knowledge about the structure of lipid bilayers has come from NMR studies of sonicated lipsomes (*31*). Unfortunately, when routine instruments are used, the signals from the inward and outward facing molecules overlap despite differences in their environment. The separation between the "outer" and "inner" signals increases when stronger magnets and Fourier transform spectroscopy are used, but even then resolution of the two signals is usually incomplete. Much better separation of the signals can be achieved by using paramagnetic ions.

The unpaired electrons of the paramagnetic ions produce a magnetic field which strongly influences other nuclei located in their vicinity. When this microscopic field is superimposed on the external magnetic field of the NMR spectrometer it can considerably exceed it in effect and may dramatically change the resonances of neighboring nuclei. According to such changes the paramagnetic ions may be divided into two classes. (i) Those such as Eu^{3+} and Pr^{3+} which have very short electron relaxation times; these cause large chemical shifts but with negligible line broadening. (ii) Those such as Mn^{2+} and Gd^{3+} which have long relaxation times and cause isotropic effects resulting in extensive line broadening.

For more than 15 years the paramagnetic ions have been used extensively in NMR studies of single organic molecules (*32*). In 1970 we started to apply them in the investigation of sonicated liposomes (*2, 33*), and immediately obtained some interesting results. In our initial experiments, salts of manganese and various lanthanides were added to sonicated egg lecithin vesicles and the resonances of the trimethylammonium protons were compared. In the presence of manganese the signal had two components: one broadened and becoming lost in the baseline and the other narrow with an integral intensity approximately 0.4 times the intensity shown by the initial sample. In the presence of lanthanides the signal split into two uneven lines: the smaller remained put, and the more intense shifted either downfield or upfield depending on the nature of the lanthanide (Fig. 1A). These effects were explained by assuming that, because of the known impermeability of lecithin liposomes for cations, only the signal from outward facing head groups was affected by the paramagnetic ions, the residual or unshifted peaks belonging to phospholipid molecules located on the inner surface of the bilayer that are not accessible to the metal. Further investigations (*19, 34*) showed that paramagnetic ions cause similar changes in the phosphorus (Fig. 1C) and the ^{13}C resonances (Fig. 1B) of the polar head group nuclei. This opened the possibility of assaying any phospholipids, irrespective of whether or not they contain choline, and of monitoring separately the perturbations of the outer and inner shells of the bilayer. Thus a number of other laboratories soon began to use shifted NMR spectra to investigate membrane structures (*35*).

One way to detect transbilayer asymmetry with the use of shift reagents is to measure the outside-to-inside intensity ratios of a given signal shown by two different components in vesicles of different component ratio and known size. Alternatively, the outside resonances may be quenched exhaustively by broadening agents and the residual peaks compared. To obtain reliable results, it is expedient to register separately the signals for each component. From the integral intensities of the separated or residual resonances it is then possible to calculate the number of molecules of each component in the outer and inner shells of the membrane.

A major difficulty in the interpretation of such NMR data is that it requires knowledge of at least three parameters: the vesicle size, the thickness of the bilayer, and the packing density of the phospholipids in the inner and outer shells. Some of these parameters can be eliminated by recording two different sets of resonances (for example, those of the phosphorus atom and those of the *N*-methyl protons), with one set of signals being used for the size determination and the other for estimating the numbers of outward and inward facing molecules. It is also possible to use simultaneously two different shift reagents, one applied from the outside and the other, causing an opposite shift, trapped in the inner space (*36*).

The main merits of the shift reagent method—accuracy, sensitivity, and simplicity—have made it valuable in the detection of lipid asymmetry. In contrast to chemical, enzymatic, and serological probes the paramagnetic ions seem not to induce serious changes in the structure of the bilayer when they are applied in low concentrations. At higher concen-

trations the ions may alter the packing density of the phospholipids and induce leakage and precipitation of the vesicles. This obstacle is not usually critical, however, because the metal may be used at very low concentrations. Thus, even less than one manganese ion per vesicle (that is, one manganese ion per 1000 phospholipid molecules, approximately) is sufficient to broaden the signal of the outward facing headgroups beyond detection (*37*). The reason for such high effectiveness is that the ions are bound only weakly to the phospholipids and exchange rapidly between various binding sites, the exchange rate being much faster than the separation of the chemical shifts of the nuclei belonging to bound and free lipid molecules.

The shift reagent method has permitted the assay of lipid asymmetry in a great variety of sonicated liposomes and has successfully been used in structural studies of serum lipoproteins (*38*). As far as we know, the method has not been applied to other natural membranous structures. This is because the method is best suited to small vesicles (with larger structures the resonances are broad and poorly resolved), and interpretation of the spectra of natural membranes is hampered by their complexity (*39*).

Phospholipase Digestion Studies

Phospholipases are hydrolytic enzymes capable of splitting phospholipid molecules at specific sites. Since the enzymes are thought to be nonpermeating it can be argued that phospholipase action on intact membranes is restricted to those phospholipids which are present on the outer surface, while phospholipase treatment of inside-out vesicles or of disrupted membranes will lead to degradation of phospholipids located on the inner surface or on both sides, respectively. One approach with this method is based on the fact that some phospholipases require specific cofactors. If phospholipases are trapped without cofactor inside resealed ghosts, and if cofactor is then added, there should follow a selective breakdown of the inside facing phospholipids. Phospholipases A_2 (E.C. 3.1.1.4) and C (E.C. 3.1.4.3) as well as sphingomyelinase (sphingomyelinphosphodiesterase; E.C. 3.1.4.2), an enzyme which specifically hydrolyzes sphingomyelin, have been used extensively to examine phospholipid sidedness, especially in erythrocytes (*40*). However, the interpretation of phospholipid digestion data is difficult because phospholipases of different origin show a great variety of action against liposomes and natural membranes. Thus, pancreatic phospholipase A_2 and phospholipase C readily degrade phospholipids in liposomes and erythrocyte ghosts, but do not hydrolyze them in intact cells (*41, 42*). Phospholipase A_2 from other sources, such as from snake or bee venoms, degrades phospholipids in intact human erythrocytes. It is difficult to decide whether such differences are caused by different penetrating abilities of the phospholipases or by differences in the lipid composition of the membranes. It has been suggested (*42*) that the ability of phospholipases to exert their action depends on the lipid packing density, and this density may be different not only in native membranes and ghosts but also in the outer and inner monolayers. Besides this, many membranes possess their own phospholipase activities which may interfere with the determination of the action of exogenous phospholipases. Furthermore, the products formed during the enzymatic hydrolysis may cause serious perturbations of membrane structure, including phase separation and phospholipid flip-flop which in turn may lead to cell lysis.

Intermembrane Phospholipid Exchange

Eukaryotic cells produce specific proteins that are capable of transferring lipids from one membrane to another (*43*). There is good reason to believe that the protein-mediated lipid exchange involves only one half of the bilayer. From this laboratory it has been reported that the treatment of sonicated liposomes with lipid-transfer proteins causes changes in the NMR signals of outward facing phospholipids, those of the inward facing molecules remaining unaffected (*44*). When lipid-transfer proteins are applied to donor membranes from the outside, only the lipids of the outer shell are readily exchangeable [the range of donor membranes studied extends from lip-

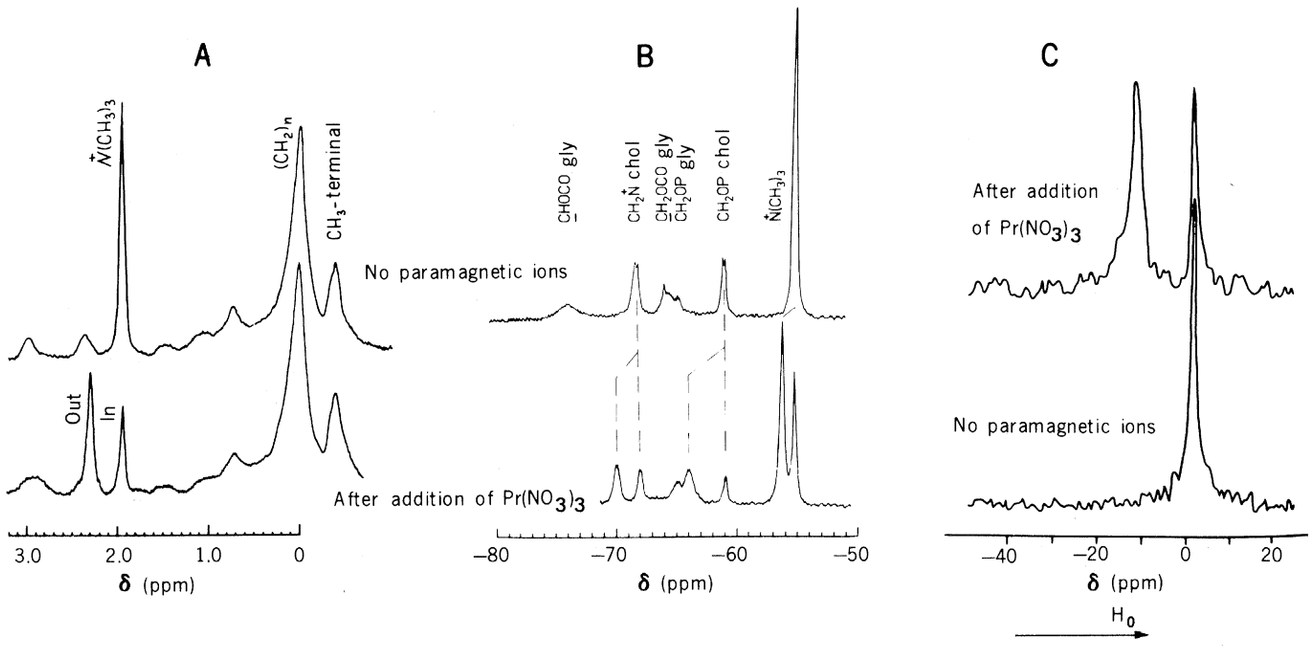

Fig. 1. Nuclear magnetic resonance spectra of sonicated lecithin liposomes. (A) Proton resonances, (B) carbon-13 resonances, and (C) phosphorus resonance. Abbreviations: *gly*, glycine; *chol*, choline.

osomes (*4, 45*) to mitochondria (*46*), erythrocytes (*6*), and bacterial protoplasts (*47*)]. On the other hand, evidence has been presented that lipids are incorporated only into the outer monolayer of the acceptor membranes (*48*). Thus shift reagent NMR revealed that protein-mediated lipid exchange between two populations of sonicated liposomes would result in the formation of highly asymmetric vesicles with no less than 90 percent of the newly introduced lipids on the outside (*44*). When spin-labeled lecithin was transferred from liposomes to mitochondrial particles the labeled lipids were almost completely located on the outer surface (*49*).

In comparison to other methods used in the study of lipid asymmetry, the exchange procedure has a number of merits. Lipid-transfer proteins are completely nonpermeating, have no lytic activity, and cause only minimal perturbations of membrane structure. A major difficulty in the interpretation of lipid exchange experiments is that in biological membranes not every phospholipid is exchangeable. Membrane phospholipids can exist either as components of the bilayer or as components of lipoprotein structures which are not in the bilayer modality. We found that the latter lipids, which probably form an intermediate phase between the proteins and the bilayer (boundary lipids), are normally much less exchangeable (*50*). Another complication is that the protein-mediated exchange is accompanied by spontaneous lipid transfer caused by fusion of the donor and acceptor membranes. This process results in the nonselective incorporation of foreign phospholipids into both halves of the membrane (*44, 49*).

Immunochemical Methods

Some phospholipids, such as acidic phospholipids—cardiolipin, phosphatidylglycerol, and phosphatidylinositol and its derivatives—as well as neutral sphingomyelin (*51*) display immunological activity. Since antibodies to phospholipids are directed against the polar head groups (*52*) they could be useful in the study of phospholipid asymmetry. Such studies have been conducted in order to locate cardiolipin and phosphatidylglycerol in mitochondria and microsomes (*53*) and phosphatidylglycerol in bacterial membranes (*54*). However, only qualitative information has been obtained by the immunochemical approach. The scope of the method is limited by the small number of serologically active phospholipids. Interpretation of the results may be complicated because the immunological reactions depend on the presence of auxiliary lipids such as lecithin and cholesterol. Besides this, cross-reactions of antibodies to cardiolipin and antibodies to phosphatidylinositol have been reported (*55*).

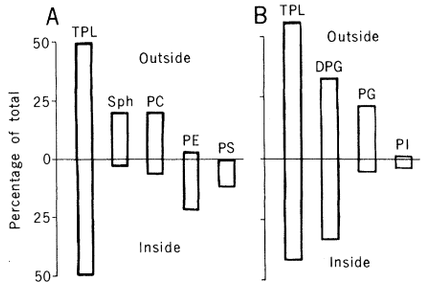

Fig. 2. Distribution of phospholipids between (A) the inner and outer layer of the human erythrocyte membrane (*41*), and (B) the inner and outer layer of the protoplasmic membrane of *M. lysodeikticus* (*47*). Abbreviations: *TLP*, total phospholipids; *Sph*, spingomyelin; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *DPG*, diphosphatidylglycerol; *PG*, phosphatidylglycerol; *PI*, phosphatidylinositol.

Transmembrane Distribution Patterns of Phospholipids

The use of shift reagents and chemical labeling revealed that sonicated liposomes composed of binary mixtures of lecithin with other lipids are usually asymmetrical. Some negatively charged phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidic acid) and the zwitterionic phosphatidylethanolamine accumulate predominantly at the inner surface (*24, 36, 56*), while other species such as the strongly acidic phosphatidylglycerol (*37*) and the zwitterionic sphingomyelin (*56*) prefer the outer surface of the bilayer. Sonicated liposomes composed of lecithin and cholesterol may also be asymmetric (*57*). If the lecithins are unsaturated, much higher cholesterol concentrations are found on the inside than on the outside of the vesicles. However, with fully saturated lecithins the component distribution becomes symmetrical.

Little is known with certainty about the quantitative distribution of phospholipids across natural membranes. Although it seems likely that cell membranes are generally characterized by asymmetrical phospholipid distributions, the evidence on this point is still fragmentary. This evidence is most substantial for the erythrocyte membrane. Chemical labeling (*14–18, 22*), phospholipase digestion (*40, 58*), and lipid exchange studies (*6*) gave consistent results showing that the outside of the erythrocyte membrane is composed mainly of cholinephosphatides (lecithin and sphingomyelin), while the aminolipids (phosphatidylethanolamine and phosphatidylserine) are confined to the inner side (Fig. 2A).

In rat liver microsomes the phospholipid distribution seems to be reversed: phospholipase digestion studies revealed a predominance of aminophospholipids on the outside and a predominance of phosphatidylcholine and sphingomyelin on the inner surface (*59*). It is thus reasonable to surmise that an enrichment in aminophospholipids of the side contacting with the cytoplasm may be a general feature of mammalian cell membranes.

Phospholipid asymmetry has been established also for some viruses. In the membrane of the influenza virus grown in bovine kidney cells, sphingomyelin was found mainly in the inner layer; phosphatidylcholine and phosphatidylinositol are enriched in the outer surface, whereas phosphatidylethanolamine and phosphatidylserine are present in similar proportions in each surface (*60*). Since these virions acquire their bilayer by budding out from the membrane of their host cells it has been proposed that the asymmetry of the viral membrane characterize the host cell plasma membrane as well [see, however (*62*)]. Another virus for which the phospholipid distribution has been elucidated is the lipid-containing bacteriophage PM2 (*25*) which grows on a marine pseudomonad. The main two phospholipids of the PM2 virus are phosphatidylglycerol and phosphatidylethanolamine (*61*). Chemical labeling showed about 10 percent of the former and 75 percent of the latter phospholipid to be accesible from the outside.

It is not possible to draw conclusions from the above data about the phospholipid distribution in the cytoplasmic membrane of the host cell because the phospholipid composition of the host and the virus are quite different (*62*). For a more simple microorganism, *Acholeplasma*, it has been suggested from results of chemical labeling that its aminophospholipids (aminoacyl esters of phosphatidylglycerol) comprising 6 percent of the total phospholipids are localized mostly on the inner surface of the plasma membrane (*16*). The cell membrane of another *Mycoplasma* species, *M. capricolum*, has been found to be asymmetric with respect to cholesterol, the greater part of the sterol being localized on the outer surface (*17*). However, the trans-

membrane distribution of the major mycoplasma phospholipids remains unknown.

The only bacterial membrane for which the quantitative distribution of all the constituting phospholipids has been elucidated is the cytoplasmic membrane of *Micrococcus lysodeikticus* (*47, 63*). This organism produces only three phospholipids: diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylinositol. The outer wall of *M. lysodeikticus* cells is easily removed by lysozyme treatment, and the surface proteins of the resulting protoplasts are almost completely destroyed by Pronase (E.C. 3.4.24.4). When such Pronase-treated protoplasts were incubated with lecithin liposomes and lipid transfer proteins isolated from rat liver, about 80 percent of the phosphatidylglycerol, 50 percent of the diphosphatidylglycerol, and 20 percent of the phosphatidylinositol were transferred from the protoplasts to the liposomes. Each of these phospholipids was digested to corresponding extent without the cells being lysed when the protoplasts were probed by phospholipases A_2 and C. From a comparison of these results with those obtained with ghosts of *M. lysodeikticus* it was concluded that diphosphatidylglycerol distributes almost evenly between the inner and outer surfaces of the protoplasmic membrane, whereas phosphatidylglycerol is located predominantly on the outer surface and phosphatidylinositol on the inner one (Fig. 2B)

Causes of Lipid Asymmetry

The primary cause of the transbilayer asymmetry of sonicated liposomes is the different curvature of the inner and outer monolayer. As a consequence of this difference, the packing densities of the lipid molecules in the two layers are different, and lipids with different charge and space requirements distribute unevenly between the two shells. On the basis of such differences one might expect that in mixtures of neutral and charged phospholipids the latter would tend to accumulate in the outer monolayer, where the intermolecular charge repulsion must be smaller (*64*). However, the fact that a number of acidic phospholipids concentrate in the inner shell (*36*) demonstrates that the distribution of phospholipids in highly curved bilayers is governed not only by charge repulsion. Probably a more important factor is the relative size of the phospholipid head group. An illustrative example is lipsomes composed of neutral lecithin and negatively charged phosphatidylserine. Having a relatively small head group, the latter phospholipids prefers the inner shell at *p*H 5.5 to 6.5; however, the degree of asymmetry depends on the component ratio, being less pronounced at higher phosphatidylserine concentrations when charge repulsion becomes more important (*36*). When the negative charge of the serine head group is increased by increasing the *p*H, the asymmetry of the bilayer first disappears and then may be inversed, phosphatidylserine accumulating in the outer shell (*56*).

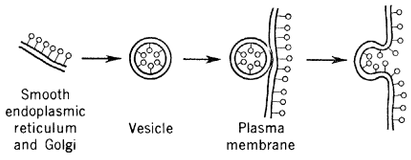

Fig. 3. Proposed mechanism for the biogenesis of plasma membranes of eukaryotic cells. Membrane synthesis is initiated in the endoplasmic reticulum leading to the formation of precursor vesicles whose fusion with already existing plasma membrane results in formation of a new plasma membrane. During the fusion process the original transmembrane asymmetry of the vesicle is inverted.

The inside-outside distribution of phospholipids in small vesicles depends not only on the charge and size of the polar headgroup but, also, to a certain degree, on the hydrocarbon chains and on the general shape of the molecules. For a small vesicle the area available for the polar head group and the hydrocarbon chains of a lipid molecule are different in the two halves of the bilayer. Thus, for a molecule at the inside of a sonicated liposome the methyl ends of the fatty acids can occupy an area 1.5 times that covered by the head group, whereas the opposite is true for a molecule on the outside of the bilayer (*65*). These considerations suggest that lipids having a polar head area greater than the area occupied by the hydrocarbon chains should better fit in the outer sphere, while molecules with a polar head smaller than the nonpolar end area should accumulate in the inner shell. Thus, lipids with identical head groups but different fatty acids can be expected to distribute asymmetrically, the more unsaturated species preferring the inner monolayer. This, probably, is the cause of the asymmetry found in sonicated liposomes composed of (unsaturated) egg lecithin and (saturated) sphingomyelin (*56*). Relief of strain may also be achieved by combining lipid molecules of different shapes. This could explain why cholesterol (small polar end) is frequently found together with lecithin and sphingomyelin, both having relatively large polar head groups. Since cholesterol is known to condense the molecular area of lecithins, especially that of the more loosely packed unsaturated species (*66, 67*), the preference of cholesterol for the inner shell in small lecithin-cholesterol vesicles may be explained by the assumption that in such vesicles tightening of the highly curved inner monolayer is thermodynamically more profitable than such action on the outer layer.

There are several other ways in which chain unsaturation may affect phospholipid asymmetry. Because of the *cis*-configuration of the double bonds, unsaturated phospholipids do not pack as closely as the fully saturated species. Consequently, in mixed vesicles composed of phospholipids with identical head groups but with different degrees of unsaturation, more unsaturated species may reside preferentially in the less compact monolayer. On the other hand, the conformation of the polar head group on the vesicle surface depends critically on the degree of unsaturation of the hydrocarbon chains (*67*), the area per head group increasing progressively with the number of double bonds (*66*). Because the packing constraint on the polar head is less restrictive for molecules residing in the outer surface, one could expect phospholipids with identical head groups and different chain saturation to distribute asymmetrically, the unsaturated species concentrating in the outer shell (*68*). Which of such different effects predominates depends on the composition of the vesicles, the hydration of the head groups, the presence of ions in the medium, and the *p*H. In any case, geometrical and electrostatic factors should become less operative as the vesicle size increases. Thus, the relatively small asymmetry in mixed phosphatidylethanolamine-lecithin vesicles (*56*) (although the head group areas of these two lipids are quite different) may be explained by the mixed vesicles being of a much larger diameter than egg lecithin vesicles [at 0.1 and 0.6 mole fractions of phosphatidylethanolamine the vesicle radii are 123 and 207 Å, respectively (*69*)].

Implications of Lipid Asymmetry

What is the relevance of the above considerations to biological membranes? It is generally recognized that metabolically active membranes involve highly folded regions with low radii of curvature. Examples are cristae in inner mitochondrial membranes, mesosomes in bacterial membranes, and the brush borders of intestinal epithelial cells. Some of

these active regions might also be the sites of membrane biogenesis or assembly. If so, the lipid asymmetry of such membranes could arise during biosynthesis at highly curved sites and might be determined by the above-mentioned thermodynamic factors.

Some membranes, such as the plasma membrane of eukaryotic cells, have rather low biosynthetic activity and do not appear to grow at specific sites. Rather, they are enriched by the random insertion of constituents. In such membranes, lipids are most probably inserted by either of two pathways: (i) fusion of the existing membrane with small intracellular vesicles, or (ii) protein-mediated lipid transfer. In the first case the original orientation of the precursor vesicles which may be determined by thermodynamic factors would be inverted in the target membrane (see Fig. 3). In the second case lipid asymmetry would be generated by the highly asymmetric mode of interaction of the lipid-transfer proteins with the donor and acceptor membranes. Experiments in vitro have demonstrated that such proteins are able not only to exchange phospholipids but also to produce a net phospholipid transfer [see (*48*)]. If the latter ability were retained in vivo, the protein-mediated lipid transfer would contribute to the asymmetry of membrane structures.

Regulation and maintenance of transmembrane lipid asymmetry may result from the combined action of some other factors such as selective flip-flop, the degradation and recombination of phospholipid molecules, and the exchange of membrane phospholipids with the surrounding medium. A number of enzymatic degradations, modifications, and interconversions are known to occur with the lipids in intact membranes. If the corresponding lipases and other enzymes are localized asymmetrically, they will affect the transmembrane distribution of the phospholipids. The possible interplay of such factors may be exemplified by a hypothesis put forward in order to explain the distribution of phospholipids in the *M. lysodeikticus* plasma membrane (*47, 63*) (see Fig. 2B). According to our hypothesis, the main membrane phospholipids (phosphatidylinositol, phosphatidylglycerol, and diphosphatidylglycerol) are synthesized on the inner surface of the membrane. The greater part of the first lipid remains inside while the last two lipids distribute between the two monolayers at the site of biosynthesis, possibly by a facilitated flip-flop mechanism (*70*). Most of the phosphatidylglycerol remaining on the inner surface is then converted to diphosphatidylglycerol by a specific enzyme which has been found to be present in the membrane of *M. lysodeikticus* (*71*).

The study of lipid asymmetry poses a number of unresolved questions which could have important implications. Asymmetric lipid compositions could regulate differential membrane fluidity in each half of the bilayer. For example, it seems quite likely that for correct functioning, the outer monolayer of a plasma membrane must be maintained in a more rigid state than the inner one. Differences in lateral compression between the two monolayers could lead to differences in head group conformations and compositional (liquid against gel) heterogeneity, which should have serious consequences for the distribution and functioning of membrane proteins. Alternatively, the higher proportion of negatively charged phospholipids on one side of the bilayer may facilitate interactions with extrinsic membrane proteins and be a necessary condition for the activity of membrane-associated enzymes.

It has been proposed that membranes whose phospholipids are distributed asymmetrically can act as bilayer couples, that is, the two halves of the bilayer can respond differentially to perturbation while remaining coupled to one another (*72*). For example, if neutral phospholipids are accumulated in the exterior and anionic phospholipids in the cytoplasmic half of a plasma membrane, it seems probable that permeable cationic agents will intercalate mainly into the inner layer expanding it relative to the exterior half. This could lead to various functional consequences including shape changes of the cell, induction of phagocytosis, and cell locomotion. This hypothesis has been utilized to explain a number of morphological changes of erythrocytes induced by drugs and electrolytes (*72, 73*). It is evident that lipid asymmetry should also be taken into account in reconstitution experiments between isolated proteins and lipids.

Interesting problems which thus far have escaped attention are connected with the microheterogeneity of membrane asymmetry. Although lateral diffusion of lipids is known to occur rapidly one could anticipate different lipid orientations in different regions of a single membrane, for example, in curved and flat areas or in the neighborhood of membrane proteins as compared to exposed bilayer regions (*74*). Such local differences should influence the accessibility and motion of receptor proteins in the plane of the membrane. Clearly, there are many more questions to answer. With the development of more sophisticated methods many of them will become amenable to definitive analysis.

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The Marine Scientific Research Issue in the Law of the Sea Negotiations

Ocean Policy Committee of the Commission on International Relations NAS–NRC.

Almost a decade has passed since Arvid Pardo, the Maltese Ambassador to the United Nations, startled the diplomatic world by his proposal that the United Nations declare the seabed and its resources "beyond the limits of present national jurisdiction" to be "The Common Heritage of Mankind" and, thus, not subject to appropriation by any nation for its sole use.

This phrase, "The Common Heritage of Mankind," rang throughout the world with great resonance. At the 25th General Assembly in 1970, after 3 years of debate, the United Nations formally adopted the concept of the oceans as "The Common Heritage of Mankind" and voted to convene, within 3 years, a Third United Nations Law of the Sea Conference to formulate an international sea law treaty that would translate this vital concept into reality.

This statement was drawn up for the Commission by Paul Fye, Woods Hole Oceanographic Institution; John Knauss, University of Rhode Island; Warren Wooster, University of Washington; and William Burke, University of Washington.

Marine Scientific Research

In the Law of the Sea negotiations, which began in 1973, delegates have been more concerned with national pride, national rights, and national resources than with the "common heritage" concept. The most notable effect of the negotiations so far, after five long and difficult sessions, has been the movement toward increased coastal state jurisdiction through the very considerable extension of national boundaries seaward. Swept up in this move to a 200-mile economic zone is the question of jurisdiction over scientific research. Customary law clearly supports complete freedom for scientific research in the water column beyond the territorial sea. In the current Law of the Sea negotiations, however, proponents of the economic resource zone concept argue that authorization for all scientific research must go along with the regulation of the exploitation of fisheries and mineral resources from the zone. The 200-mile economic zone encompasses approximately 37 percent of the ocean area (Fig. 1).

Nature knows no artificial boundaries. Ocean phenomena do not stop at nation-