Membrane Asymmetry

The nature of membrane asymmetry provides clues to the puzzle of how membranes are assembled.

James E. Rothman and John Lenard

There is now compelling evidence that biological membranes are vectorial structures; that is, their components are asymmetrically distributed between the two surfaces. This provides a molecular basis for functional asymmetry. For proteins and carbohydrates, the asymmetry is absolute. Every copy of a polypeptide chain has the same orientation in the membrane. Plasma membrane carbohydrates, whether bound to protein or to lipid, are external. However, lipid asymmetry is not absolute; almost every type of lipid is present on both sides of the membrane bilayer, but in different amounts.

Membrane asymmetry is maintained by an exceedingly low frequency of spontaneous movement of constituents from one surface to the other, and this lack of transmembrane diffusion in formed membranes provides an important clue to the mechanisms of membrane assembly by focusing our attention on a fundamental problem: How are membrane macromolecules incorporated into the external side of the membrane when the source of energy and biosynthetic precursors is on the inside, in the cytoplasm? The difference in the nature of lipid and protein asymmetry indicates that different mechanisms exist to solve this topological problem for the two principal components of biological membranes.

In this article, we first discuss the available evidence for membrane asymmetry and how it is maintained. The primary emphasis is on lipid asymmetry, since the results are more recent, and the topics of protein and carbohydrate asymmetry have been extensively reviewed (*1–3*). We then consider the implications for the mechanisms by which cells assemble asymmetric membranes.

Asymmetry of Proteins

The transmembrane disposition of the membrane proteins of erythrocytes has been thoroughly investigated (*3*). The fact that the only membrane of the mature red cell is the plasma membrane, the ability to produce leaky cell ghosts which can also be resealed (*4*), and the development of methods for producing nearly homogeneous preparations of subcellular vesicles with either normal or inverted sidedness (*4*) have all made the erythrocyte an exceedingly favorable object for study. Several experimental approaches have led to satisfying agreement on the arrangement of the major red cell membrane polypeptides. Externally localized residues of a protein can be demonstrated by the availability of the protein in intact cells, resealed ghosts, or right-side-out vesicles to externally added proteases (*5–7*), enzymatically catalyzed iodination (*8*), or nonpenetrating reagents (*5, 9–14*). Table 1 describes most of these treatments. Residues of proteins on the cytoplasmic side of the membrane can be modified only in unsealed ghosts or in inside-out vesicles. By these criteria, all but two of the major proteins of the erythrocyte membrane are exposed only at the cytoplasmic surface (*3*). The two other major components differ in that they are glycoproteins which are exposed at both surfaces.

A polypeptide that can be modified from both sides of the membrane may span the lipid bilayer asymmetrically, or it may be symmetrically arranged (*15*). These alternatives are readily distinguished by determining whether different sequences of the polypeptide are exposed at the inside and outside surfaces. This can be most convincingly shown by subjecting the modified polypeptide to peptide mapping (*10*). By this criterion, the two glycoproteins mentioned above, called glycophorin and band 3, span the membrane, and most or all copies of these chains have the same orientation (*7, 10*). Indeed, the complete amino acid sequence of glycophorin has been determined (*16*). The amino terminal portion of this glycoprotein is outside, the carboxy terminal portion is inside, and the segment that passes through the lipid bilayer is hydrophobic.

Thus, the major red cell membrane proteins either span the membrane asymmetrically or are exposed only at the cytoplasmic surface. No protein has been found to be symmetrically distributed or to be unexposed on either surface (*3*). More recent results (*17, 18*) with enveloped animal viruses, mitochondria, endoplasmic reticulum, platelets, sarcoplasmic reticulum, lymphocyte plasma membrane, intestinal brush border, and bacterial membranes all indicate an absolute asymmetry in the orientation of proteins in these membranes.

Protein asymmetry is maintained by the exceedingly low rate at which it can decay. For a large membrane protein to invert its orientation, many polar and charged groups would be forced to pass through the hydrocarbon core of the lipid bilayer (*19*). This does not happen. Indeed, if proteins were capable of transmembrane motion on the time scale of labeling experiments (up to an hour), asymmetry would not have been detected. Consistent with this finding, membrane transport proteins do not function by transmembrane rotations (*2*). Instead, they are asymmetric transmembrane proteins (*18, 20–22*).

Asymmetry of Carbohydrates

The observation that secreted proteins are generally glycosylated while cytoplasmic proteins are not (*23*) led to the expectation that plasma membrane carbohydrates would be found associated exclusively with extracellular portions of membrane components. The earliest experimental data in support of this notion were those of Eylar and co-workers (*24*), who found that all of the sialic acid of red cells could be removed with neuraminidase. Subsequently, it was shown that most red cell sialic acid is linked to the external (*25*) amino terminal portion (*16*) of glycophorin. Indeed, all of the carbohydrate contained in glycophorin is external to the cell (*16, 25*). The carbohydrate portions of band 3 and the minor

James Rothman is a postdoctoral fellow in the Department of Biology, Massachusetts Institute of Technology, Cambridge 02139, and was a graduate student in the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, when this article was written. John Lenard is a professor of physiology at the College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway 08854.

erythrocyte glycoproteins are all externally localized (*7, 26*).

Further support for the external localization of membrane oligosaccharides comes from the work of Singer and colleagues (*27*), who used ferritin derivatives of lectins [plant proteins which bind to specific sugars (*28*)] to stain the surface carbohydrate of red cells, several mammalian plasma membranes, and organelle membranes from myeloma cells for electron microscopy. They found that only the outside surface of plasma membranes and the inner, extracytoplasmic surface of intracellular membranes were stained.

The sugar-containing portions of the membrane glycoproteins of enveloped animal viruses are external to the virion lipid bilayer (*17*). These viral-coded proteins are almost certainly synthesized, glycosylated, and processed through the normal, cellular pathways. The wide variety of animal cells in which viruses can replicate provides an additional indication that all protein-bound carbohydrate in animal cell plasma membranes is externally localized. The large literature on the biological effects of external lectins on animal cells (*28*) supports this conclusion.

Evidence for the external localization of glycolipids (*1*) is less abundant. Glycolipids of intact erythrocytes can be labeled by reduction with [^{3}H]borohydride after galactose oxidase treatment (*7, 26*), but the glycolipids of inside-out vesicles cannot (*7*), permitting the conclusion that red cell glycolipids are completely external. Similar studies with enveloped viruses also demonstrated external glycolipid (*29, 30*).

The asymmetry of protein carbohydrate is maintained by the protein asymmetry. There are, as yet, no measurements of the rate of transmembrane movement of glycolipids. However, the bulky water-soluble sugar residues should make this process even slower for glycolipids than for phospholipids.

Table 1. Agents used to characterize membrane asymmetry.

Agent	Functional group of compound	Reference
Neuraminidase	Terminal sialic acid residues	(*24*)
Galactose oxidase + NaB^3H_4	Terminal galactose and *N*-acetyl galactosamine	(*7, 26*)
Diazotized sulfanilic acid	Primary amine, protein side chains	(*5, 9*)
Isocyanostilbenedisulfonic acids	Primary amine	(*12*)
Formylmethionyl (sulfonyl) methyl phosphate (FMMP)	Primary amine	(*10*)
Trinitrobenzenesulfonic acid (TNBS)	Primary amine	(*7, 11*)
Isethionyl acetimidate (IAI, nonpenetrating), ethyl acetimidate (EAI, penetrating)	Primary amine	(*14*)
Pyridoxal phosphate + NaB^3H_4	Primary amine	(*13*)
N-(4-azido-2-nitrophenyl)-2-aminoethane-sulfonate	Nonspecific	(*15, 107*)
Lactoperoxidase + $Na^{125}I$	Tyrosine residues	(*8*)
Proteolytic enzymes	Peptide bonds	(*5–7, 10*)
Phospholipases	Phospholipids	(*35, 36, 43*)
Phospholipid exchange proteins	Phospholipids	(*37, 43*)
Vesicles, uncatalyzed exchange	Cholesterol	(*46, 47*)

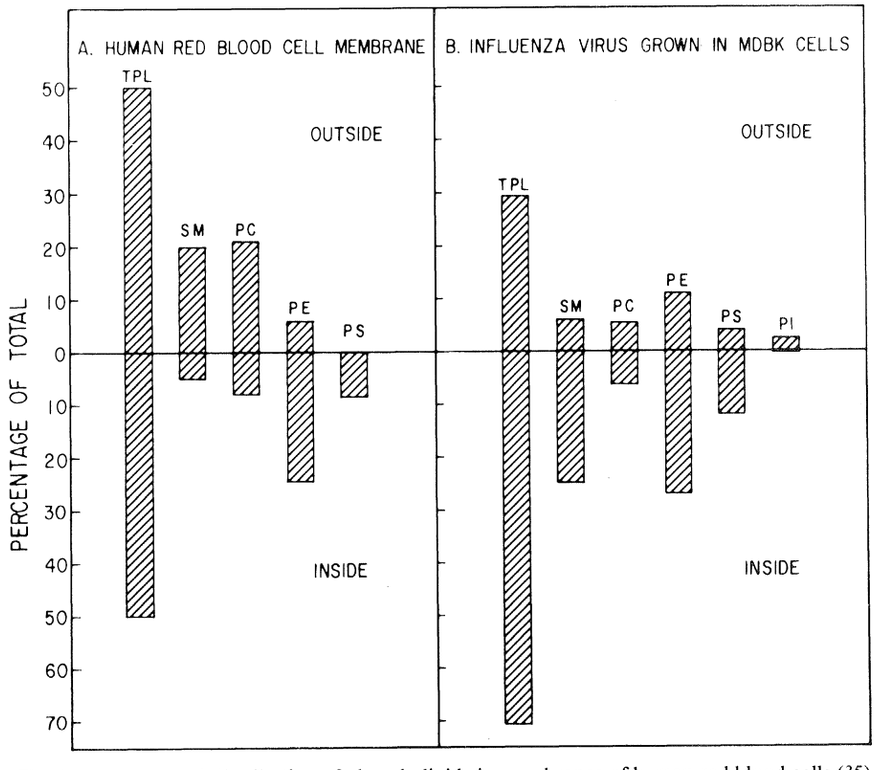

Fig. 1. Asymmetrical distribution of phospholipids in membranes of human red blood cells (*35*) and influenza virus (*43*) grown in MDBK cells, expressed as mole percent. Abbreviations: *TPL*, total phospholipid; *PC*, phosphatidylcholine; *SM*, sphingomyelin; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; and *PI*, phosphatidylinositol.

Lipid Asymmetry in Red Cells

The idea that lipids of red cells are organized in a bilayer is now more than 50 years old (*31*), but it is only recently that it was recognized by Bretscher (*32*) that different lipids comprise the two monolayers. Based, in part, on his observation that phosphatidylethanolamine reacted much more readily with FMMP (Table 1) in red cell ghosts than in whole cells, Bretscher (*32, 33*) proposed that the aminophosphatides, phosphatidylethanolamine and phosphatidylserine, are mainly in the cytoplasmic monolayer of the bilayer, and he inferred that the choline derivatives, phosphatidylcholine and sphingomyelin, the remaining major phospholipids, comprise the external monolayer. Several objections that were initially raised to this conclusion were satisfactorily answered by subsequent work (*14, 34–37*). Bretscher (*33*) measured only the initial velocity of reaction. Thus, it was possible that phosphatidylethanolamine was symmetrically distributed, but merely more reactive on the cytoplasmic surface. However, it was found that much more total phosphatidylserine and phosphatidylethanolamine were modified by TNBS in unsealed ghosts than in cells (*34*). Another criticism was that the membrane might be altered during the formation of ghosts so as to reveal sites that were unreactive in intact cells. This objection was eliminated by experiments (*14*) in which cells and ghosts were completely amidinated with

the analogous pair of penetrating and nonpenetrating imidoesters, EAI and IAI. The penetrating reagent modified aminophosphatides to the same extent in intact cells as in unsealed ghosts. The nonpenetrating reagent modified ghosts to a much greater extent than whole cells.

External red cell phospholipids have also been identified by their susceptibility to phospholipases (*35, 36*). Van Deenen and co-workers (*35*) found that 48 percent of the total phospholipid of red cells could be hydrolyzed without hemolysis (Fig. 1). Specifically, 76 percent of the phosphatidylcholine, 82 percent of the sphingomyelin, 20 percent of the phosphatidylethanolamine, and none of the phosphatidylserine were degraded, confirming the external localization of the choline phosphatides that was inferred (*32, 33*) from the labeling studies. The phospholipid of unsealed ghosts was completely degraded under the same conditions (*35*). A similar study of rat erythrocytes (*36*) showed that 44 percent of the total phospholipid could be degraded in intact cells. This pool contained all of the sphingomyelin, 62 percent of the phosphatidylcholine, 20 percent of the phosphatidylethanolamine, and about 6 percent of the acidic phospholipids, phosphatidylserine and phosphatidylinositol. The fatty acid composition of lipids with the same polar head group was the same on both sides of the membrane (*36*).

The membrane is, of course, altered by treatment with reagents or phospholipases. The unlikely possibility that these harsh treatments lead to artifactual conclusions about the distribution of lipids can now be set to rest. Thus, 63 to 75 percent of the phosphatidylcholine of rat erythrocyte ghosts was found to be in the external monolayer, on the basis of its accessibility to phospholipid exchange protein (*37*). This protein makes it possible to study the distribution of phospholipids without covalent modification of the membrane.

Taken together, these findings provide compelling evidence that lipid bilayer asymmetry is a general feature of red blood cell membranes. This asymmetry is of a simple kind (*38*), with aminophosphatides almost exclusively inside and choline phosphatides mainly outside.

The distribution of cholesterol, the major neutral lipid of red cells, has not been established with certainty, although it has been reported that cholesterol is present in both surfaces in large amounts (*39*). This is expected since cholesterol is nearly equimolar with total phospholipid in red cells, and artificial phospholipid bilayers do not readily incorporate cholesterol in larger than equimolar amounts (*40*).

Experimental Aspects

Does lipid asymmetry exist in membranes other than those of erythrocytes? Unfortunately, many other membranes are less ideally suited to determine this. Therefore, it may be useful to consider some general factors that permit meaningful studies of lipid asymmetry.

Purity. It is crucial that all the lipids in the test sample can be assigned to the membrane under study and not to an impurity. Purification must be achieved at some point in the analysis. Most plasma membrane preparations from complex cells have not been demonstrated to be sufficiently free of contaminating membranous material to be useful for this purpose. In fact, most criteria for the "purity" of a membrane preparation are functional ones, and therefore not appropriate for studies of lipid asymmetry; an example is the absence of a contaminating enzyme activity. The criterion of purity is most easily met by the choice of well-characterized systems, such as mammalian red cells, enveloped viruses, and certain bacteria, where microscopic analysis shows that the original structure contains only one membrane.

Closure. All membranes must be present as closed vesicles.

Unique sidedness. All membrane vesicles must have the same overall sidedness. The examination of isolated membranes from complex cells involves cell disruption, and some systems, such as endoplasmic reticulum and plasma membrane, are fragmented and then reseal (*41*). The sidedness of resealing must be clearly established.

Asymmetric analysis. It is important to show that putative nonpenetrating reagents do not cross the membrane under study. This cannot be inferred from previous studies with the same reagent but with other membranes. For example, the rate at which TNBS enters erythrocytes (*11, 34*) and bacterial cells (*42*) depends strongly on experimental conditions. Enzymes and exchange proteins are of sufficient size that penetration through the membrane would appear unlikely, except through processes such as phagocytosis or membrane rupture.

Localization of inaccessible lipids. The possibility that some externally localized lipids are not available to external probes must be eliminated. Ideally, the inner and outer surfaces should be examined separately by the same probe. This can be accomplished if membranes can be prepared as vesicles with either a right-side-out or an inside-out orientation. In most cases, this is not yet possible.

An alternative, but less satisfactory, solution is to investigate the accessibility of all membrane lipids: phospholipids, glycolipids, and cholesterol. If the fraction of total lipid accessible is comparable to that expected on the basis of the geometry of the membrane, it can safely be concluded that few if any lipids are inaccessible. It is particularly useful in these cases to employ several independent probes.

Uniform labeling. When the method of asymmetrical analysis involves radioactive lipids, it is essential that all pools of lipid have the same specific radioactivity.

Lipid Asymmetry in Other Membranes

There are now a sufficient number of studies that meet the criteria enumerated above to permit an evaluation of the generality of lipid asymmetry.

Influenza virus has been thoroughly studied. Like many other enveloped animal viruses, influenza obtains its lipid bilayer by budding out from the plasma membrane of the host cell (*17*). In the process, viral nucleocapsids envelop themselves with a modified region of plasma membrane, which contains host cell lipids in closely conserved proportions, but which has only viral-coded proteins. Thus, the mature virion is a suitable object for study of the transmembrane distribution of lipids since it contains a single lipid bilayer whose sidedness presumably reflects that in the host cell plasma membrane, but in any case is the same for all particles. The viral particles are readily purified and can be labeled uniformly by growing the host cells for several generations in radioactive medium.

The distribution of phospholipids in influenza virus was determined by using two phospholipases and two different phospholipid exchange proteins as probes for external phospholipids (*43*). Exchange proteins, found in the cytoplasm of most or all eukaryotic cells, reversibly extract a specific phospholipid molecule from a membrane to form a soluble, diffusible complex, thereby catalyzing the one-for-one exchange of phospholipids between membranes (*44*). Only the phospholipids of the external monolayer of sealed vesicles can be ex-

changed (*45*). Thus, exchange proteins can be exploited to distinguish external from internal phospholipids with little or no perturbation of membrane structure.

Two types of phospholipid asymmetry (*43*) are evident in influenza virus grown in bovine kidney cells (Fig. 1). First, there are unequal amounts of total phospholipid on the two sides of the membrane. About twice as much is inside as outside. Also, the phospholipid composition of the two sides is markedly different. The asymmetry found in influenza virus differs in both of these respects from that found in red blood cells. In particular, sphingomyelin is mainly in the internal layer of the viral membrane, and aminophosphatides constitute a much larger fraction of the external phospholipids.

The distribution of viral glycolipid (*30*) and cholesterol (*46*) has also been investigated. Independent access to the inner surface of the viral envelope was not possible, so it was necessary to examine all viral lipids. About half of the viral cholesterol was available for exchange (*46*). By analogy with studies performed with model membranes (*47*), it was concluded (*46*) that this pool represents external viral cholesterol. It is clear from Fig. 1 that if two-thirds of the total phospholipid is internal, with the cholesterol distributed almost symmetrically across the bilayer, then much of the outer surface is left to glycolipids. Indeed, an exceptionally large amount of glycolipid has been found in virus grown on the cell line employed in these studies (*30, 48*), and these glycolipids appear to be externally localized (*30*). When the independently determined distributions of phospholipids, glycolipids, and cholesterol are tallied together with their relative abundances in the virion, the distribution of total lipid that results is compatible with the geometry of the viral bilayer (*43*).

Bacterial cells offer another suitable experimental system for the study of lipid asymmetry. The gram-positive bacilli are particularly favorable, since they contain only one major membrane system, the cytoplasmic membrane (*49*). The transmembrane distribution of phosphatidylethanolamine in *Bacillus megaterium* has been determined with IAI and TNBS (*42*). Only 33 percent of the total phosphatidylethanolamine could be modified by these reagents under conditions where these agents did not enter cells. Unavailable phosphatidylethanolamine was internally localized, since TNBS could modify only 67 percent of the phosphatidylethanolamine of inside-out vesicles under the same conditions. From the predominantly internal localization of phosphatidylethanolamine, it was inferred (*42*) that most of the other major bacterial phospholipid, phosphatidylglycerol, is externally localized.

The distribution of the phospholipids in the membrane of bacteriophage PM2 has been the subject of another investigation (*50*). It was found that most of the phosphatidylglycerol was in the external monolayer, and that most of the phosphatidylethanolamine was restricted to the inner half of the bilayer, as in *B. megaterium*. Inferences about the distribution of phospholipids in the host bacterium could not be drawn since the lipid composition of the phage is grossly different from that of its host (*50*).

These results firmly establish lipid asymmetry as a general property of biological membranes. However, they also make it clear that there is no unique distribution of lipids. The type of asymmetry is quite variable from one membrane to another. Indeed, there can be great variability even in one system. For example, two different influenza virus membranes with different phospholipid compositions have strikingly different distributions of phospholipids (*43*).

Lipid asymmetry differs from that of proteins and carbohydrates. While the latter two membrane constituents are distributed across the membrane in an absolute, all-or-none manner, each phospholipid is generally present on both sides of the membrane, albeit in different amounts.

Maintenance of Lipid Asymmetry

The problem of how lipid asymmetry is maintained cannot be resolved without a knowledge of the rate at which the lipid molecules diffuse across the membrane through the lipid bilayer. This process is termed flip-flop (*51*) and, since it is a diffusion event, it is spontaneous and randomizes the phospholipids between the two sides of the bilayer.

Measurements of flip-flop depend on the ability to create a lipid asymmetry in a membrane and then to follow the rate at which the asymmetry disappears. The modifications of membrane components that are required to establish the lipid asymmetry should be innocuous ones, such as isotopic substitutions (*52, 53*).

Isotopic phospholipid asymmetries can be created by replacing radioactive phospholipids of the external monolayer with unlabeled phospholipids, using the phospholipid exchange proteins. This has been achieved with artificial phospholipid vesicles (*45*) and with influenza virions (*43, 46*). In neither case could flip-flop be detected; only lower limits for the half-time of this hypothetical process could be estimated. These lower limits were 11 days for phosphatidylcholine in vesicles, and 10 days for phosphatidylcholine and 30 days for sphingomyelin in influenza virus membranes.

Other methods have been devised for examining flip-flop in model membranes. Phospholipid vesicles containing phosphatidylcholine and phosphatidylethanolamine were modified with IAI to convert the external phosphatidylethanolamine to its amidine derivative. The subsequent appearance of internal phosphatidylethanolamine on the external surface was assayed. None could be detected over the time course of the experiment; a lower limit of 80 days for the half-time of this process was estimated (*54*). In another approach (*55*) a planar bilayer film separating two aqueous compartments was formed from monolayers, one composed of phosphatidylethanolamine, the other of phosphatidylserine. The resulting asymmetry of electrostatic charge produced a membrane with an asymmetric current-voltage curve. This asymmetry was maintained completely for the lifetime of the film, about 10 hours. Significantly, application of a membrane potential of 100 millivolts did not promote phospholipid flip-flop.

The lack of detectable flip-flop of phospholipids can be explained, in part, by the high activation energy that would be required to bring the polar group through the hydrocarbon core of the bilayer (*19*). Steric constraints are almost certainly responsible for the exceedingly slow rate of flip-flop of cholesterol. On the basis of polarity alone, rapid flip-flop of cholesterol might have been expected. However, flip-flop could not be detected when radioisotopic cholesterol asymmetries were created by exchange techniques in phosphatidylcholine-cholesterol vesicles (*47*) or in influenza virus membranes (*46*). Estimated lower limits for the half-time of cholesterol flip-flop were 6 and 3 days, respectively. Cholesterol is known to restrict the mobility of neighboring phospholipid fatty acyl chains, creating an ordered surface layer (*56*), which may prevent cholesterol flip-flop because of its low lateral compressibility.

Even though flip-flop is immeasurably slow in pure lipid bilayers and viral membranes, it is possible for transmembrane movement to occur without flip-flop—that is, without the polar head group of the phospholipid passing through the hydrocarbon core of the bilayer. From this point of view, it is intriguing that trans-

membrane movement of phosphatidylcholine has been observed in whole erythrocytes (*36*) and erythrocyte ghosts (*37*). The half-time for the loss of a radioisotopic asymmetry is only a few hours in these membranes. This rate is, conservatively, about three orders of magnitude faster than flip-flop in phospholipid vesicles (*45, 54*) and influenza virus membranes (*43, 46*). Further, recent evidence (*57*) indicates a considerably more rapid transmembrane movement of phosphatidylethanolamine in growing bacterial membranes. In this system a radioisotopic asymmetry, introduced biosynthetically during growth, is dissipated in about 3 minutes, indicating a rate of transmembrane movement at least five orders of magnitude faster than flip-flop of phosphatidylethanolamine in a lipid bilayer (*54*). These striking differences in rate are most plausibly explained (*1, 43*) if a membrane protein serves a catalytic function, providing a route through the membrane alternative to flip-flop. A transmembrane protein could provide this route by means of an intramembranous surface with which the phospholipid head groups could interact during passage between the two aqueous surfaces of the membrane. Further, the differences in rates between bacterial and red cell membranes might suggest the involvement of specific proteins (*1*). Rapid transmembrane movement appears to be essential for the assembly of membrane bilayers.

These findings have not helped to clarify the question of what maintains lipid asymmetry in biological membranes. In principle, the asymmetrical distribution could be either stable or unstable (*58*). A stable asymmetry would be restored if an arbitrary displacement of phospholipids across the membrane was introduced, and might or might not require metabolic energy for its maintenance. An unstable asymmetry would not be restored. A stable asymmetry requires transmembrane movement, and the lack of such movement during the lifetime of the membrane or its constituents is sufficient evidence that the asymmetry is unstable. Hence, protein asymmetry in general and lipid asymmetry in viral and model membranes are unstable. However, lipid asymmetry in red cells and bacteria could be either stable or unstable. Rapid transmembrane movement would be incompatible with unstable asymmetry unless that movement were highly specific, involving only one-for-one exchange of identical molecules (*54*) across the membrane. Such an exchange could take place at any rate without affecting asymmetry.

Role of Lipid Asymmetry

The functional significance of lipid asymmetry is still obscure. One possibility that has been suggested is that polar group asymmetry, in conjunction with variations in the fatty acid constituents among lipid classes, results in different fluidities for the two monolayers (*1*). This is very unlikely for plasma membranes, which contain large amounts of cholesterol; indeed, spin label measurements of the fluidity of the two sides of erythrocyte membranes by Devaux and colleagues (*53*) indicate no such differences. However, for membranes where cholesterol is absent or present in small quantities, such as bacterial membranes and many organelle membranes of higher cells (*59*), differences in fluidity remain a real possibility (*60*).

Specific lipids could fulfill the specific requirements of membrane-bound enzymes whose lipid binding sites are asymmetrically distributed. However, these requirements should be satisfied by only small quantities of the specific lipids (*61*), so that an asymmetrical distribution of the bulk lipid should not be necessary.

Separation of electrostatic charge produced by the asymmetrical distribution of anionic phospholipids has been successfully used to explain many of the effects of membrane-active drugs on the shape of erythrocytes (*62*). It is also possible that regions of acute curvature, which are common in membranes of complex cells, are produced by a local lipid asymmetry that gives the inner and outer monolayers different surface tensions (*58, 63*).

In the absence of any positive evidence for a functional role, the view that lipid asymmetry serves no biological purpose, but instead is an incidental consequence of the topological constraints imposed on the mechanism of biogenesis of external lipids (see the section on origins of lipid asymmetry), must be given serious consideration.

Origins of Protein Asymmetry

Membrane proteins can be classified (*19*) as integral or peripheral according to whether or not they interact with the hydrocarbon core of the lipid bilayer. The localization of cytoplasmic peripheral membrane proteins must be determined by the fact that they are made inside the cell and simply cannot leave.

The mechanisms by which integral membrane proteins acquire their absolute asymmetry are, no doubt, more complex. It is useful to attempt to distinguish two types of integral proteins, which may be called "ectoproteins" and "endoproteins" (Fig. 2). Ectoproteins have substantial hydrophilic mass which projects beyond the extracytoplasmic surface of the lipid bilayer (extracellular in the case of the plasma membrane, intracisternal in the case of the endoplasmic reticulum). Examples of ectoproteins are the glycoproteins, which form the spikes of enveloped animal viruses (*17*); histocompatibility antigens (*64*); glycophorin (*16*); and the intestinal brush border hydrolases (*64*). Endoproteins do not project beyond the extracytoplasmic surface of the bilayer, and may have most of their mass associated with the cytoplasmic side of the membrane. Pos-

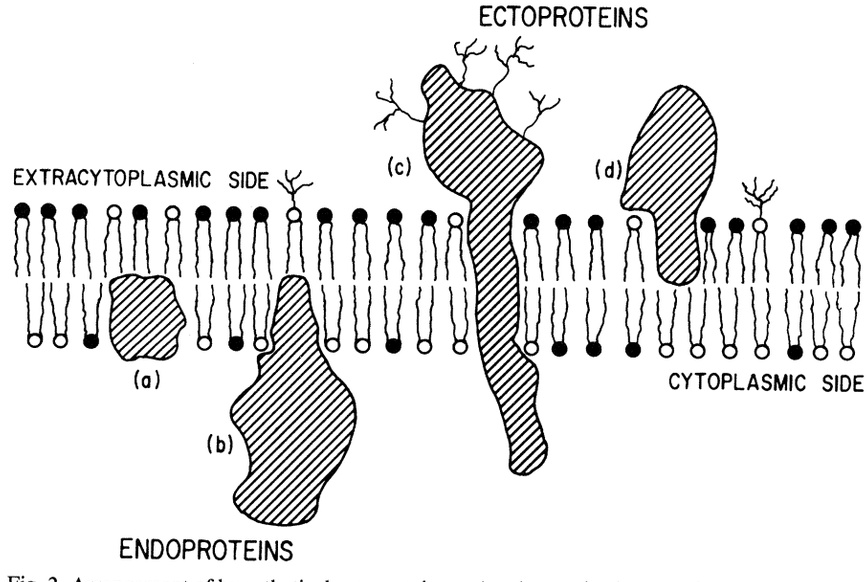

Fig. 2. Arrangement of hypothetical ectoproteins and endoproteins in a membrane (see text): (a and b) endoproteins; (c and d) ectoproteins.

sible endoproteins include cytochrome b_5, cytochrome b_5 reductase (*65*), and other constituents of the cytoplasmic side of the endoplasmic reticulum (*66*). We will explore the hypothesis that *the differences in the membrane topography of endoproteins and ectoproteins require different mechanisms for integration into the membrane, and may lead to different sites for the biosynthesis and ultimate intracellular localization of these two kinds of integral membrane proteins.*

An obvious but complex problem that has long been recognized (*67*) for secreted proteins is also posed by the existence of ectoproteins: How does the extracytoplasmic portion of the ectoprotein reach the extracytoplasmic side of the membrane (*68*)? It does not seem plausible to suppose that the folded, hydrophilic portion of an ectoprotein could spontaneously cross the lipid bilayer—it is precisely the slowness of this process that stabilizes protein asymmetry in formed membranes. The extent of the problem can be appreciated by considering certain viral membrane glycoprotein spikes (*17*), ectoproteins whose extracytoplasmic portion protrudes about 130 Å outward from the surface of the viral bilayer—more than three times the width of the bilayer itself. Therefore, it seems that the extracytoplasmic portions of ectoproteins must pass through the permeability barrier of the membrane in an unfolded form, presumably during their polymerization on ribosomes (Fig. 3). Since ectoproteins are synthesized on only one side of the membrane, and the extracytoplasmic portion crosses the bilayer during biosynthesis and cannot cross back after synthesis, ectoproteins must have an all-or-none asymmetry.

On these general grounds, one expects that ectoproteins are synthesized on ribosomes that are bound to membranes by the nascent chains, if by no other interaction. This assertion is strongly supported by the finding that the messenger RNA's that encode the membrane glycoproteins of vesicular stomatitis and Sindbis viruses are attached to ribosomes that are associated with membranes (*69, 70*). Indeed, the ribosomes that make the Sindbis viral membrane proteins can be released from the membrane only in the presence of puromycin (*70*), an antibiotic that dissociates nascent polypeptides from ribosomes.

It has been appreciated for many years that there are two functionally distinct classes of ribosomes in higher cells (*71*): membrane-bound and free ribosomes. Membrane-bound ribosomes are known to manufacture secreted proteins (*71*) and have been suggested (*72, 73*) to be a source of membrane proteins, while free ribosomes are known to produce proteins for the cytosol.

The dynamic relationship between membrane-bound and free ribosomes has been greatly clarified by the recent work of Blobel and his colleagues (*74*). They achieved in vitro secretion of proteins into ribosome-free vesicles, derived from endoplasmic reticulum, by adding these vesicles to a conventional protein-synthesizing system containing free ribosomes that was programmed with messenger RNA for a secreted protein. This showed that membrane-bound ribosomes are derived from the pool of free ribosomes. When globin messenger was used, globin chains were synthesized but were not segregated into the vesicles. Furthermore, these workers discovered that the proteins secreted by the pancreas have a hydrophobic amino terminal sequence of 16 residues, which is cleaved by an enzyme of the endoplasmic reticulum before chain completion.

These findings were predicted by the "signal hypothesis" (*74, 75*), which proposes that during polypeptide synthesis an amino terminal "signal sequence" of the growing chain emerges from the large subunit of a free ribosome and directs the ribosome to specific receptor sites on the endoplasmic reticulum membrane, which bind the signal sequence and permit secretion. Nascent proteins, such as globin, whose ultimate destination is the cytoplasm do not carry such signal sequences.

When the ribosome attaches to the membrane, a junction capable of secretion is formed. To account for the passage of hydrophilic sequences across the bilayer, it was further proposed (*74*) that this junction consists of a protein-lined tunnel spanning the bilayer through which the growing chain is extruded during translation, amino terminus first. This process of "vectorial discharge" (*76*) of the nascent secreted protein into the lumen of the endoplasmic reticulum during polypeptide synthesis is well documented (*77*).

It seems likely (*74*) that the membrane-bound ribosomes that synthesize ectoproteins are, like secretory ribosomes, derived from free ribosomes that were directed to receptors of the appropriate, specific membrane by signal sequences on nascent ectoproteins (*78*). Further,

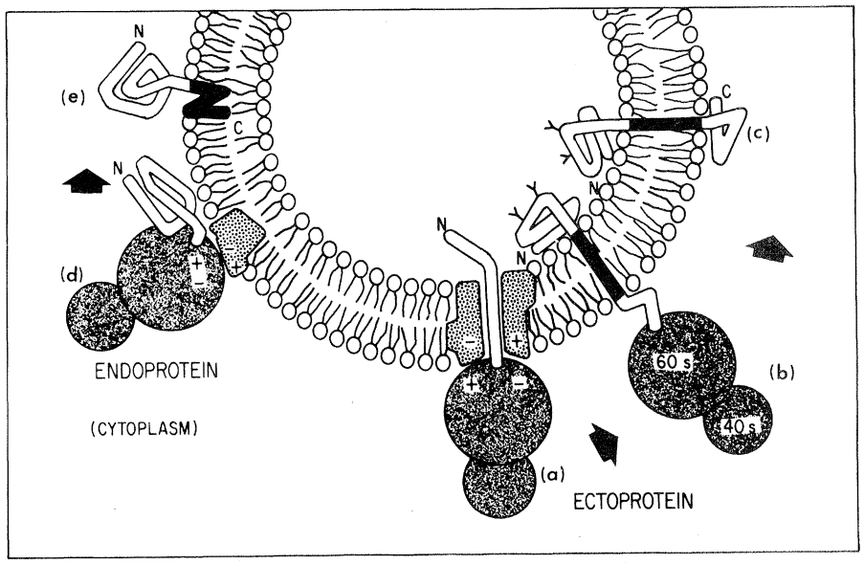

Fig. 3. Different mechanisms for the integration of ectoproteins (a to c) and endoproteins (d and e) into membranes. It is proposed (see text) that the extracytoplasmic portions of ectoproteins cross the bilayer during biosynthesis, whereas endoproteins do not. This difference is illustrated here for a typical ectoprotein (such as glycophorin) and endoprotein (such as cytochrome b_5). In (a), the ectoprotein passes, amino terminus first, through a specialized ribosome-membrane junction. Later (b), the junction ceases to function in order to prevent complete secretion, giving rise to a domain structure. The mainly hydrophobic, lipid-binding domain, located in the bilayer, is darkened. Finally (c), the ribosome has completed the cytoplasmic domain, and the transmembrane ectoprotein has been asymmetrically integrated into the membrane. In (d), an endoprotein is manufactured by a membrane-bound ribosome, but is not discharged across the membrane. The ribosome is not bound to the membrane by the nascent chain, since the carboxy terminal lipid-binding site (e) of the cytochrome b_5 molecule has not yet emerged from the ribosome. Endoproteins might also be synthesized by free ribosomes (see text). The amino (*N*) and carboxy (*C*) termini of polypeptides are marked, and the large (60*S*) and small (40*S*) subunits of ribosomes are indicated. The folding of the polypeptide chains is arbitrary, and the messenger RNA molecules have been omitted for simplicity.

since the problem of transporting portions of ectoproteins to the extracytoplasmic space is similar to that for secreted proteins, the two ribosome-membrane junctions probably function analogously. However, ectoprotein integration and protein secretion would have to differ in one fundamental respect. Whereas secreted proteins are extruded completely across the membrane, transmembrane ectoproteins like glycophorin (with its extracellular amino terminus and intracellular carboxy terminus) can be only partially extruded. This suggests that synthesis of transmembrane ecotoproteins occurs by a two-step process (Fig. 3). The ribosome-membrane junction through which ectoprotein extrusion occurs ceases to function before the polypeptide chain is completely extruded in these cases (possibly as the result of specific, internal sequences in the nascent ectoprotein that are lacking in secreted proteins), leaving the carboxyl region on the inside.

The two-step synthesis of transmembrane ectoproteins would necessarily produce a domain structure consisting of an extracytoplasmic domain, which is synthesized first (in the simplest case), and an intramembrane, and possibly cytoplasmic, domain (or domains), which is synthesized subsequently. These domains would then correlate directly with linear sequences of the chain—the amino terminal region with the extracytoplasmic domain, and the carboxyl terminal region with the intramembrane and cytoplasmic domain. Such a linear domain structure has now been described for several ectoproteins (*16, 64*).

In contrast to the special mechanisms that are apparently required to meet the severe topological constraints on the biosynthesis of ectoproteins, the situation for endoproteins could, in principle, be much simpler, since the biosynthesis and final destination of the hydrophilic portion of the protein are on the same side of the membrane. Thus, endoproteins could be made on free ribosomes (*1, 73, 79*) and simply diffuse to the membrane, where they would insert spontaneously in a functional form. Ectoproteins cannot do this. The physical properties of some endoproteins are certainly compatible with this possibility. For example, pure cytochrome b_5 can be added to isolated endoplasmic reticulum in the absence of detergents. The exogenous cytochrome inserts into the membrane and functions indistinguishably from endogenous cytochrome (*65*). Because insertion takes place from only one side of the membrane, and since the hydrophilic domains of endoproteins cannot cross the bilayer, an absolute transmembrane asymmetry results.

However, a problem of specificity arises—cytochrome b_5 can even insert spontaneously into synthetic phospholipid vesicles and function normally (*65*) but is not found in all intracellular membranes (*80*). If such an endoprotein were, in fact, synthesized on free cytoplasmic ribosomes, an additional mechanism would be required to ensure specific insertion into the appropriate membrane (*81*). This could be achieved if the endoprotein were synthesized in the cytoplasm as a precursor incapable of inserting into any membrane. This precursor could be activated by proteolytic cleavage (*73*) by an enzyme of the target membrane. For example, phospholipase A_2 can interact with membranes only after it has been cleaved from its prophospholipase form to reveal a hydrophobic amino terminus (*82*). Indeed, the importance of posttranslational cleavage is becoming increasingly evident (*83*).

Alternatively, specific insertion of an endoprotein would be achieved if this protein were synthesized on a ribosome attached to the target membrane (*84*) so that the first membrane encountered by the new endoprotein would be the correct one (Fig. 3). Presumably, such a nascent endoprotein would direct its ribosome to the specific membrane with a signal sequence. The attachment site could not permit the nascent endoprotein to be discharged through the membrane, or an ectoprotein would result. Therefore, this junction would have to be distinct from that formed by ribosomes that bear nascent ectoproteins (Fig. 3). In this connection, it is interesting to point out that there exists a class (up to 40 percent of the total) of active membrane-bound ribosomes that can be released from the endoplasmic reticulum membrane in the presence of high concentrations (0.5*M*) of KCl (*85*). These loosely bound ribosomes do not manufacture secreted proteins or ectoproteins, since they are not anchored to the membrane by the nascent chains (*86*). However, this class of ribosomes could be a major source of endoproteins. In fact, cytochrome b_5, an endoprotein, is attached to the endoplasmic reticulum membrane by a hydrophobic segment at its carboxy terminus (*65*), and it has been suggested (*84*) that it is made on membrane-bound ribosomes. Such ribosomes would be expected to be loosely bound in the sense that they would elute from the membrane at high salt concentrations (Fig. 3) since they would not be hydrophobically anchored to the membrane by the nascent cytochrome b_5 molecule.

In pointing out well-characterized proteins whose structures seem clearly to classify them as either endoproteins or ectoproteins, we have not discussed a potentially significant class of membrane proteins that cannot yet be classified on the basis of topography alone. The best example is bacteriorhodopsin, a transmembrane protein whose polypeptide chain spans the membrane several times, but which does not protrude far above the lipid bilayer on either side (*18*). Presumably, however, its biosynthetic route, once known, will serve to place it unambiguously in one of these two general classes of integral membrane proteins. Indeed, we believe the differences between the biosynthetic pathways provide the fundamental distinction between endoproteins and ectoproteins.

Intracellular Processing

In many cases integral membrane proteins require transport from their site of synthesis to their ultimate destination (*27, 79, 87–90*). For example, envelope glycoproteins of animal viruses are first detected in intracellular membrane fractions. At later stages, after up to 30 minutes, these proteins appear in the plasma membrane (*79, 87, 89, 90*). This elaborate intracellular processing sequence, during which glycosylation occurs (*79, 87, 88*), is consistent, in a general way, with a mechanism for the formation of the plasma membrane from endoplasmic reticulum originally envisioned by Palade (*91*) on the basis of the intracellular pathway of secreted proteins (*88*). It seems clear that protein asymmetry is preserved during the intracellular transit of membrane proteins between organelles. It has been proposed that this is achieved by a series of fusion processes that conserve the sidedness of membranes (*27, 88*), so that the intravesicular surface of intracellular membranes would be topologically equivalent to the extracellular surface of the plasma membrane.

The actual mechanism of intracellular transit has remained an enigma (*88*). One particularly vexing problem has been that certain components of the endoplasmic reticulum (such as viral glycoproteins) are transported, while others (such as cytochrome b_5 and ribosome-binding sites) are left behind. In this regard, it may be noteworthy that the integral proteins of the plasma membrane that have

thus far been characterized extend through the bilayer and into the cytoplasm (*3, 16, 64, 92*). Interaction of underlying cytoskeletal elements with this cytoplasmic segment has been suggested for the integral proteins of the red cell membrane (*93*). Furthermore, a similar interaction of cytoskeleton with transmembrane proteins can explain the effects of drugs that disrupt the cytoskeleton on the capping of external surface receptors in more complex animal cells (*93, 94*). This raises the intriguing possibility that the actual movement of transmembrane proteins from intracellular membranes to the plasma membrane is dependent on similar interactions with cytoskeletal structures. Interaction of transmembrane proteins with cytoskeleton while in the endoplasmic reticulum would automatically serve to sort them out from the noninteracting proteins of the endoplasmic reticulum, such as cytochrome b_5, which remain with this organelle (*80*). To achieve this, clustering of transmembrane proteins within the plane of the membrane would appear to be required before transfer of these special regions of the endoplasmic reticulum membrane. These regions would give rise to vesicular structures that would permit transfer of membrane without a loss of asymmetry (*27, 88*). Secreted proteins, of course, could not be transported by this mechanism, nor could ectoproteins that do not span the membrane (*95*).

Ectoproteins and endoproteins could even be distinguished on the basis of their participation in intracellular transport and processing. Atkinson and coworkers (*79*) distinguished two classes of proteins on the basis of the kinetics of their integration into HeLa cell plasma membranes. Proteins of one type were incorporated into the surface membrane almost immediately after their synthesis. These could be endoproteins or peripheral proteins that are manufactured on free ribosomes (*73*), since few, if any, ribosomes are associated with plasma membranes (*59, 96*). Another population, plasma membrane glycoproteins, began to appear in the surface membrane 12 minutes after polypeptide synthesis and fucosylation. The intracellular pool of membrane protein was membrane-bound. We suggest that this class of proteins is composed entirely of transmembrane ectoproteins (*95*). Indeed, the only proteins thus far known to be transported are ectoproteins (*87, 89, 90*).

A similar but more detailed picture emerges from studies of the assembly of the viral-coded proteins of vesicular stomatitis virus into the plasma membrane of infected cells (*79, 87*). The glycoprotein of this virus, a transmembrane ectoprotein (*97*), arrives at the plasma membrane about a half-hour after its polypeptide portion is synthesized (*79, 87*) by ribosomes of the endoplasmic reticulum (*69, 87*). During this interval, the glycoprotein is transported from its site of synthesis to other intracellular membranes, and it is further glycosylated (*87*). Glycosylation is apparently complete some 10 minutes before the glycoprotein reaches the cell surface (*87*). In contrast, the other viral membrane protein, the nonglycosylated M protein, becomes associated with the plasma membrane almost immediately after synthesis (*79*), but it is initially found in the cytoplasm (*87*), where it is synthesized by free ribosomes (*69*). The M protein appears to be an endoprotein because it is resistant to proteolysis in intact virions, and detergents are required to dislodge it from the viral bilayer (*17*).

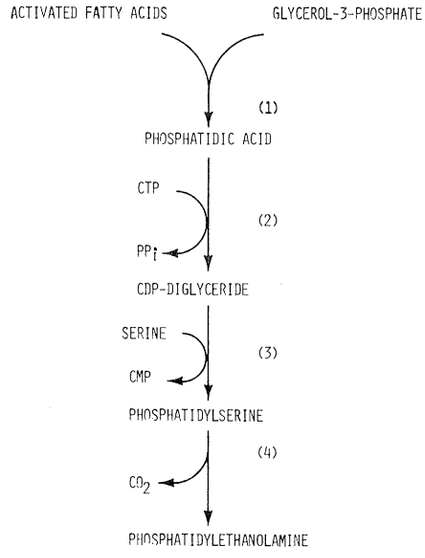

Fig. 4. Pathway for biosynthesis of phosphatidylethanolamine in *Escherichia coli* (*99*).

Origins of Lipid Asymmetry

The synthesis of lipids of the external monolayer and of the extracellular portions of ectoproteins poses similar topological problems. The source of energy and biosynthetic precursors used in the synthesis of both of these membrane components is found on the opposite (cytoplasmic) side of the membrane. However, constraints on possible solutions to this problem for lipids and proteins are quite different. Proteins are linear macromolecules, which are sufficiently long to extend across the lipid bilayer during their polymerization on ribosomes. Lipid molecules span only half the bilayer; it would seem that they are synthesized in association with one monolayer or the other.

This topological constraint is most dramatically evident in bacterial cells, where lipid biosynthesis on the cytoplasmic side of the bilayer (*1, 98*) is strongly suggested by the extensive involvement of water-soluble precursors found in the cytoplasm. This is well illustrated by the pathway for the biosynthesis of phosphatidylethanolamine in *Escherichia coli* (Fig. 4) established principally by Kennedy and his co-workers (*99*). This pathway [except for step 3 (*100*)] is catalyzed by proteins of the cytoplasmic membrane (*101*). The first three steps, resulting in the formation of phosphatidylserine, all utilize water-soluble molecules found in the cytoplasm, such as cytidine triphosphate, glycerol-3-phosphate, and serine, indicating that phosphatidylserine formation takes place in an aqueous milieu on the cytoplasmic surface of the bilayer.

The decarboxylation of phosphatidylserine to produce phosphatidylethanolamine, step 4, is the last step in the pathway and the first that does not require a cytoplasmic precursor. The decarboxylase is an integral membrane protein (*102*), and it is known from studies of temperature-sensitive phosphatidylserine decarboxylase mutants (*103*) that the synthesis of at least 95 percent of the cellular phosphatidylethanolamine depends upon the activity of this one Gene product (*104*). If this enzyme were oriented asymmetrically in the bilayer, as would be expected, it would appear that phosphatidylethanolamine could be formed only on one side of the bilayer, presumably the cytoplasmic side where phosphatidylserine must be made.

The topography of assembly of lipid bilayers in *B. megaterium* has been investigated (*57*). Surface labeling with TNBS was used to distinguish internal from external phosphatidylethanolamine. When cells were pulse-labeled by the addition of phosphorus-32–labeled inorganic phosphate or tritiated glycerol to their growth medium, all of the newly synthesized, radioactive phosphatidylethanolamine was found in the internal pool—that is, the pool not modified by TNBS. It was concluded that this phospholipid is synthesized exclusively on the cytoplasmic side of the membrane.

The extremely slow flip-flop of phospholipids documented earlier then makes it difficult to understand how newly synthesized lipids of the cytoplasmic monolayer can reach the outer half of the membrane on a time scale compatible with the rapid rate of bacterial growth

(*98*). Indeed, when pulse-labeled cells of *B. megaterium* were subsequently transferred to nonradioactive medium to determine the fate of the cytoplasmically synthesized phosphatidylethanolamine, this phospholipid was observed to equilibrate with external phosphatidylethanolamine with a half-time of about 3 minutes. This rapid rate of transmembrane movement is probably the result of a special mechanism involving membrane proteins used in membrane assembly (see the section on maintenance of lipid asymmetry).

It can therefore be anticipated that membranes capable of growth, such as bacterial membranes and, perhaps, endoplasmic reticulum membranes of animal cells (but see below), should have rapid rates of transmembrane movement (*1*) representing a special mechanism used in membrane assembly, and not flip-flop. On the other hand, membranes incapable of growth, such as viral membranes, should lack such a mechanism, and therefore might be expected to have exceedingly slow rates of transmembrane movement, limited to the inherent rate of flip-flop.

A pool of each translocated phospholipid exists on both sides of a growing membrane. The size of the pool on the cytoplasmic side would reflect the rate of translocation relative to net production, but must be of some finite size. It follows that translocated lipids cannot exhibit an absolute asymmetry. On the other hand, a lipid that is not translocated should be found exclusively in the cytoplasmic monolayer.

A translocation step could also explain, in a general way, why lipids are asymmetrically distributed, but not in a unique manner. The relative rates of synthesis and translocation of a particular lipid would be expected to vary from cell to cell, just as different cells have grossly different lipid compositions (*105*), differences which themselves depend on variations in the activities of biosynthetic and catabolic enzymes. Only in the special case where the rate of translocation is precisely one-half the rate of synthesis would a symmetric lipid bilayer result. If there were no selective pressure for a unique distribution of lipids then a variety of asymmetric bilayers would be expected for different cells, and possibly even for the same cell type grown under different conditions.

The topological constraints on lipid biosynthesis may be less demanding in eukaryotes than in bacterial cells. In animal cells, lipids are synthesized in intracellular membranes, primarily the endoplasmic reticulum (*106*), whose cytoplasmic and extracytoplasmic surfaces are both within the cell. In principle, this could permit biosynthetic steps involving water-soluble intermediates to take place on both sides of the membrane, thereby eliminating the absolute requirement for a translocation process, although it would not rule out such a process. Despite the fact that lipid biosynthesis in animal cells has been extensively studied (*106*), there is as yet no information regarding the sidedness of these reactions. Furthermore, the means by which intracellular phospholipids are conveyed to the cell surface is unknown, although membrane flow (*88*) and phospholipid exchange proteins (*44*) have been proposed as possible mechanisms. Phospholipid asymmetry of plasma membranes of animal cells could be introduced after the intracellular biosynthesis of phospholipids during their transport to the cell surface if special regions of the endoplasmic reticulum differing in lipid composition from the bulk (say, enriched in sphingomyelin and cholesterol) were transferred. This could account for the striking difference in lipid composition of plasma and endoplasmic reticulum membranes (*59*).

If some lipids of intracellular membranes of animal cells were in fact synthesized on both sides of the membrane from soluble precursors without translocation, the absolute asymmetry of membrane proteins would seem to require a separate set of enzymes for each surface. It is therefore interesting to note that multiple pathways for the biosynthesis of the same phospholipid are known in animal cells (*106*), whereas these apparent duplications have not been observed in bacteria (*99, 103*).

Conclusions

1) Membrane proteins exhibit an absolute asymmetry. No portion of a polypeptide chain is present on both sides of the permeability barrier. This asymmetry is maintained by the lack of transmembrane movement of membrane proteins during their lifetime in the membrane.

2) Two types of integral membrane proteins, called ectoproteins and endoproteins, can be distinguished on the basis of their topography and differ in their sites of biosynthesis and ultimate localization in cells. Ectoproteins are made by ribosomes bound to membranes in order to permit the extracytoplasmic, hydrophilic portions of these proteins to cross the lipid bilayer during biosynthesis. Endoproteins can, potentially, be synthesized by either free or membrane-associated ribosomes, since the hydrophilic domains of these proteins need not cross the bilayer.

3) Absolute asymmetry of integral membrane proteins results during insertion because each polypeptide chain is synthesized only by ribosomes on one side of a membrane and, in particular, because the extracytoplasmic portions of ectoproteins cross the bilayer only during polypeptide synthesis.

4) Ectoproteins, like secreted proteins, are suggested to have specific amino terminal signal sequences, which direct free ribosomes bearing nascent ectoproteins to a complementary receptor in the appropriate target membrane. These ribosomes, now membrane-bound, extrude the extracytoplasmic portions of the ectoproteins across the membrane through a specialized ribosome-membrane junction during polypeptide chain elongation. Endoproteins would not possess this sort of signal sequence.

5) To account for the incomplete secretion of transmembrane ectoproteins, it is suggested that they are synthesized by a two-step process in which the ribosome-membrane junction is altered during synthesis. This automatically gives rise to a domain structure correlated directly with the linear amphipathic nature of the amino acid sequence.

6) It is further proposed that ectoproteins and endoproteins of the endoplasmic reticulum membrane are synthesized by ribosomes attached to the membrane in distinct ways. Ectoproteins appear to be manufactured by ribosomes that are anchored to the membrane by nascent chains. Endoproteins may be made by ribosomes that are not anchored by nascent chains. These endoproteins, therefore, would require carboxyl terminal hydrophobic sequences for their insertion into the bilayer.

7) We suggest that transmembrane ectoproteins destined for the plasma membrane are transferred from their site of synthesis, the endoplasmic reticulum, in membrane vesicles specifically by the interaction of the cytoplasmic segments of the transmembrane proteins with cytoskeleton. This interaction would automatically serve to sort out these ectoproteins from the proteins of the endoplasmic reticulum that are not transferred by clustering the transmembrane proteins in the plane of the membrane before the formation of vesicles from these regions.

8) Cytoplasmic peripheral proteins and endoproteins of plasma membranes may be manufactured by free ribosomes and are not processed through the endoplasmic reticulum. The kinetics of assem-

bly of plasma membrane proteins appears to reflect these differences.

9) The asymmetrical distribution of lipids is not absolute. In general, each type of lipid is present on both sides of the membrane in some proportion. Although every membrane studied has an asymmetrical distribution of lipids, the details are highly variable. There is no ubiquitous distribution of lipids.

10) Spontaneous diffusion of lipid molecules across membranes through the lipid bilayer (flip-flop) has not been observed.

11) Transmembrane movement of phospholipids in red cell membranes and in bacterial membranes has been observed. This process occurs much faster in bacteria than in red cells, and is thought to arise from a special protein-catalyzed mechanism, distinct from flip-flop, which operates in membrane assembly.

12) Phospholipid biosynthesis is restricted to the cytoplasmic half of bacterial membranes, where the biosynthetic enzymes have access to required water-soluble cytoplasmic precursors. After synthesis, phospholipids are translocated to the outer half of the membrane bilayer.

13) Outer monolayer lipids are derived from lipids of the cytoplasmic monolayer, so that a pool of each translocated lipid must be found on both sides of the bilayer. This precludes an absolute asymmetry for lipids.

Summary

The components of biological membranes are asymmetrically distributed between the membrane surfaces. Proteins are absolutely asymmetrical in that every copy of a polypeptide chain has the same orientation in the membrane, and lipids are nonabsolutely asymmetrical in that almost every type of lipid is present on both sides of the bilayer, but in different and highly variable amounts. Asymmetry is maintained by lack of transmembrane diffusion. Two types of membrane proteins, called ectoproteins and endoproteins, are distinguished. Biosynthetic pathways for both types of proteins and for membrane lipids are inferred from their topography and distribution in the formed cells.

Note added in proof. A cell-free system has now been developed which permits the mechanisms of membrane protein assembly to be studied (*108*). The membrane glycoprotein of vesicular stomatitis virus has been synthesized by wheat germ ribosomes in the presence of rough endoplasmic reticulum from pancreas. The resulting polypeptide is incorporated into the membrane, spans the lipid bilayer asymmetrically, and is glycosylated (*108*). The amino terminal portion of this transmembrane protein is found inside the endoplasmic reticulum vesicle, while the carboxyl terminal portion is exposed on the outer surface of the vesicle. Furthermore, addition of the glycoprotein to membranes after protein synthesis does not result in incorporation of the protein into the membrane in the manner described above (*108*). Consequently, protein synthesis and incorporation into the membrane must be closely coupled. Indeed, using techniques to synchronize the growth of nascent polypeptides, it has been shown (*109*) that no more than one-fourth of the glycoprotein chain can be made in the absence of membranes and still cross the lipid bilayer when chains are subsequently completed in the presence of membranes. These findings demonstrate directly that the extracytoplasmic portion of an ectoprotein can cross the membrane only during biosynthesis, and not after.

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