



Membrane Structure: Some General Principles

Author(s): Mark S. Bretscher

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lithology as the earth's, where an eight-point sampling grid would be grossly inadequate. The lunar farside and poles remain to be sampled, but the magnesium, aluminum, and silicon concentrations on the farside, determined from orbit by x-ray fluorescence measurements, indicate that soils there are similar to the materials directly sampled in terra regions on the nearside.

4. The use of the terrestrial rock names anorthosite, norite, and troctolite is a reference to chemical similarities, and does not imply an origin in deep-seated igneous bodies as do the terrestrial names. Anorthositic rocks contain

more than 65 percent plagioclase; norites and troctolites contain roughly equal amounts of plagioclase and a mafic mineral. The latter is chiefly orthopyroxene in the case of norites, olivine in the case of troctolites. The acronym KREEP refers to potassium, rare-earth elements, and phosphorus. Our category of KREEP-poor norites and troctolites corresponds to the very-high- Al_2O_3 basalts described by P. W. Gast (in 2, p. 275).

5. One gamma equals 10^{-5} gauss.

6. With respect to the axis of rotation C is the moment of inertia, M is the mass of the moon, and R is the moon's radius.

7. The per mil notation is customarily used in studies of stable isotopes to express small differences between isotopic abundances in the sample studied and in a standard sample. Thus

$$\delta^{13}\text{C}_{\text{PDB}} = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right] \times 1000$$

where PDB refers to the Pee Dee belemnite limestone standard. Similarly, $\delta^{15}\text{N}_{\text{air}}$ expresses the isotope ratio $^{15}\text{N}/^{14}\text{N}$ relative to that in air; $\delta^{34}\text{S}_{\text{CD}}$ compares the isotope ratio $^{34}\text{S}/^{32}\text{S}$ to that in troilite (FeS) from the Canyon Diablo meteorite.

Membrane Structure: Some General Principles

Membranes are asymmetric lipid bilayers in which cytoplasmically synthesized proteins are dissolved.

Mark S. Bretscher

My purpose in writing this article is to indicate how the constituent parts of cellular membranes are organized and to suggest what this may tell us about their function and assembly. There are many excellent reviews that consider membrane proteins, carbohydrates, and lipids, but these usually cover only one of these components (1). In a sense that is unfortunate because each plays an important role in the function of the membrane. Much of what I have to say centers around the erythrocyte membrane. It is sometimes argued that the erythrocyte is dead and that therefore its membrane may be somewhat atypical. This may be true; but once it was alive, and there is much to be gained by studying fossils.

The principal components of membranes are lipids and proteins. The amount of carbohydrate is usually small, from none to less than 10 percent of the mass of the membrane. Lipids usually account for around 40 percent by weight, the balance being protein. I shall assume that the matrix of the membrane is composed of lipid molecules arranged in a bimolecular leaflet as originally proposed by Gorter and Grendel (2) and later emphasized by Danielli and Davson (3); this as-

sumption may not be acceptable to everyone, but the weight of firm evidence seems to be strongly in favor of this structure.

I first summarize what is known of three of the major protein components of the erythrocyte membrane, for each is a good example of a different class of membrane protein. This summary is followed by a consideration of asymmetry of lipid and carbohydrate distribution in membranes, and some general comments on membrane structure. As a guide to background reading, a few of the many models produced over the last 10 years is depicted in Fig. 1.

Proteins

The introduction of sodium dodecyl sulfate (SDS) as a solubilizing agent for insoluble molecules has revolutionized the study of membrane proteins. The great advantages of this detergent are that (i) polypeptides exist free from one another and from lipid molecules, presumably as micelles, and (ii) these polypeptides can be separated on a semimicro scale on SDS-polyacrylamide gels (4) where they

migrate according to their molecular weights (5). The fractionated polypeptides are readily visualized by a variety of stains. The simplicity and resolution of this system make it a most powerful technique.

When applied to erythrocyte membranes, the result is remarkably simple (6-11). About a third of all the protein resides in a pair of close bands (molecular weights in excess of 200,000); roughly another quarter is found in a band characterized often by being rather diffuse (molecular weight, about 100,000; hereafter referred to as component *a*); the remaining weaker bands vary in size (some nine components, including residual globin, with molecular weights between 90,000 and 15,000) and intensity. Scrutiny of the gels reveals many more bands, and there must be very many which, by their paucity, are not seen at all. This would include, for example, the Na^+ , K^+ -activated adenosine triphosphatase present only in a few hundred copies per cell. Those proteins visualized are present in at least hundredfold greater amounts. The proteins that I shall discuss are therefore the major components.

Even simpler is the picture seen if the gels are stained for carbohydrate instead of protein. Here just one major and several minor components are seen. These are all glycoproteins. Their molecular weights cannot be ascertained from the gels because of the large proportion of carbohydrate they carry (12). Attempts to make such calculations (13) have no theoretical basis and as such should be mistrusted.

Several proteins, then, are present in the erythrocyte membrane. How is each located with respect to the lipid bilayer? There are two distinct methods for determining which of them may be exposed on the outer surface

The author is a member of staff of the cell biology division, Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, England.

of the cell. The first is to see which polypeptides (in the SDS gel) are affected if the red blood cells are treated with a proteolytic enzyme such as pronase. This procedure, introduced by Bender, Garen, and Berg (9), relies on the assumption—so good an assumption that we might regard it as fact—that pronase cannot get inside the cell. Only exterior proteins can be degraded. The second method is based on the use of a chemical reagent that can label proteins, but whose solubility properties prevent its diffusing through the lipid bilayer into the cell. In this way only external proteins are labeled. This method was originally that of Maddy (14) who used a fluorescent reagent (stilbene-4-acetamido-4'-thiocyano disulfonate or SITS), but was not followed up. Since then a handful of radioactive reagents has appeared: these enable one to see which proteins on an SDS gel become labeled. The first of these was developed by Berg (6) (^{35}S -labeled diazobenzene sulfonate), followed by ^{35}S -labeled formylmethionyl sulfone methyl phosphate (10) and, more recently, pyridoxal phosphate (15). A different approach has been developed by Phillips and Morrison (8); they mixed labeled iodide with lactoperoxidase and hydrogen peroxide. An I^+ -lactoperoxidase complex is presumably generated, and this labels tyrosine and histidine residues on surface molecules. The specificity of this reaction is in some doubt in that other workers have also found extensive labeling of lipids.

One of the most gratifying facts about the erythrocyte membrane is that both general approaches—proteolytic enzymes or chemical labeling—show that just two of the major components seen on an SDS gel are located at the external surface of the membrane. Both the glycoprotein and component *a* are cleaved by pronase; other proteins are not (9). Both these proteins are labeled from the exterior; others are not (10, 16).

One might then ask where all the other proteins are located. At present we have no means of determining this other than to prepare membranes or "ghosts" of red blood cells. This is most frequently done by lowering the ionic strength of the medium some fivefold, so that the cells lyse, hemoglobin is released, and empty sacs of the cells can be isolated. Some of these ghosts reseal; many do not. Such a ghost preparation can be used to look

at both sides of the membrane, most effectively by the labeling approach. When ghosts are labeled, every protein component is seen to become labeled. No protein is inaccessible to labeling from both sides of the membrane and so totally buried within the bilayer. We therefore see that almost all the protein of the erythrocyte ghost is associated with the inner, cytoplasmic, surface of the membrane.

There is one little caveat in this last conclusion—one often voiced, once even printed (17). How do we know that lysis of the cell has not altered the structure of the membrane, that some rearrangement has not occurred? In a gross sense the membrane has been ruptured. But to imagine that some extensive molecular rearrangement has occurred is unreasonable. After all, membrane enzymes still seem perfectly functional, and the process of lysis must be one of the most gentle that biochemists traditionally do to the molecules they study. Not surprisingly, different methods of looking at the cytoplasmic side of the erythrocyte

(18) which involve different methods of lysis yield the same general picture. It seems to me that arguments about detailed structural changes that may occur on lysis of erythrocytes are red herrings, unless there is *good* evidence for them. None exists to my knowledge.

Component *a*

This large protein (molecular weight about 100,000) is the only major protein exposed on the surface of erythrocytes. Treatment of red cells with trypsin, while cleaving the glycoprotein, leaves component *a* apparently intact. However, it is sensitive to pronase in intact cells and is degraded quantitatively. A new band is seen on SDS-polyacrylamide gels to arise from this cleavage, and I call this component *c*. It has a molecular weight of about 70,000 and is a part of component *a* resistant to further digestion. The digested portion of the original component *a* has so far remained elusive. It has been reported that compo-

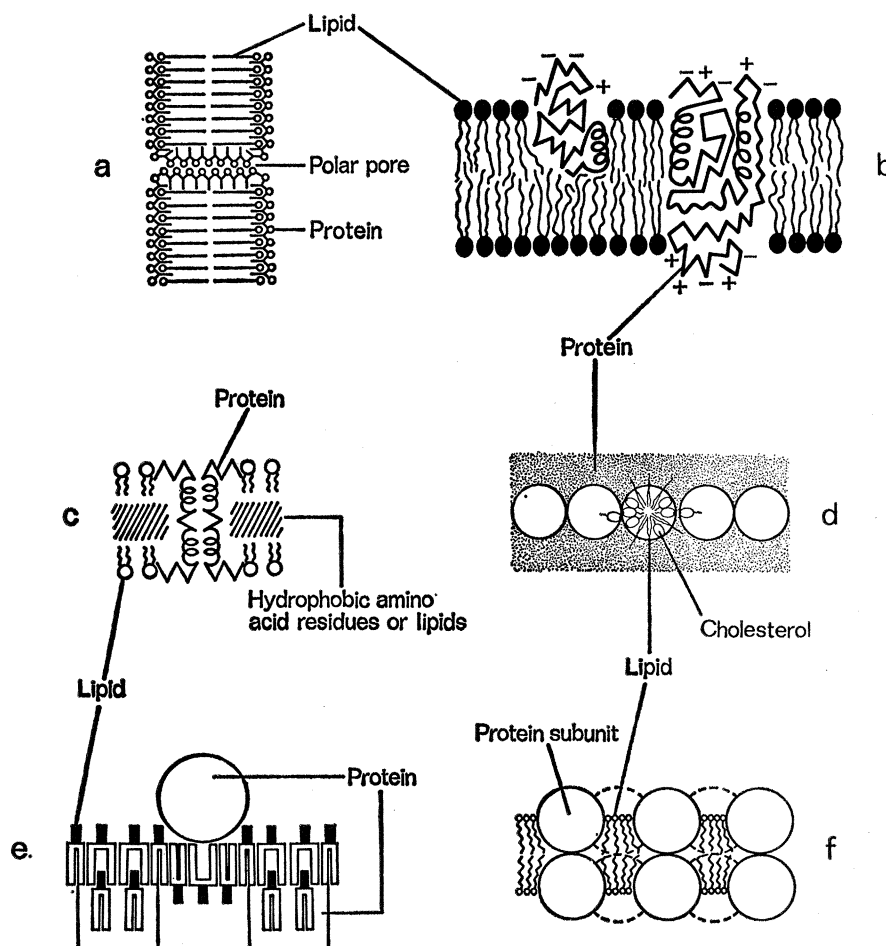


Fig. 1. Various membrane models. (a) Goldup, Ohki, and Danielli (3a). (b) Singer and Nicolson (69). (c) Lenard and Singer (70). (d) Lucy (71). (e) Kreutz (72). (f) Vanderkooi and Green (73).

nent *a* carries a small amount of carbohydrate, around 10 percent (11). Such a low level of carbohydrate would probably not seriously affect the apparent molecular weight. It is quite possible that component *a* has lipid covalently associated with it, but there is no evidence.

The fact that all copies of component *a* are degraded by pronase indicates that over the time average of such an experiment (perhaps an hour), every molecule has been available to the external protease. Component *a* is labeled by radioactive reagents added from the cell exterior. When component *a* is labeled in cell ghosts, more of the polypeptide is accessible to label by the reagent (19). This is shown by two-dimensional peptide patterns of component *a* labeled in either intact cells (from the outside) or ghosts (from both sides). The latter peptide pattern is about twice as complex as the former and this shows that part of this molecule extends across the bilayer. The very fact that the peptide patterns derived from the external and internal surfaces are different shows that component *a* does not rotate across the membrane.

The picture we then have of this protein is that of a large folded polypeptide straddled across the bilayer. The fact that only very limited digestion of it occurs from the cell exterior by pronase suggests that not much of the protein is available on the outer surface of the cell. This conclusion is matched by the great simplicity of the two-dimensional pattern of labeled peptides derived from the cell exterior. Furthermore, the peptide pattern of that part of component *a* derived from the cytoplasmic surface of the membrane is also simple: in an experiment in which amino groups are preferentially labeled, only some ten radioactive peptides were contributed from the cytoplasmic side. All this suggests that most of component *a* is buried in the membrane. In other words, it probably has a roughly globular shape, with its head above, and feet below, the lipid bilayer.

Is component *a* a structural protein, or does it have a specific enzymatic function? My guess is that it has some catalytic role, and, more specifically, may be an anion channel. The main physiological function of the erythrocyte membrane is to enable bicarbonate and chloride ions to equilibrate across it (20). The measured chloride flux across the erythrocyte membrane

is approximately 2.4×10^{11} ions per second (at 37°C in 120 mM Cl⁻) (21). Since some 5×10^5 copies of component *a* exist per cell (10) this would give a minimum turnover number of about 5×10^5 ions per second per channel, if component *a* is the channel. [It should be noted, however, that this number is considerably smaller than the proposed flux for the sodium channel (22).] The anion channel is partially inactivated by reagents that modify amino groups from the cell exterior and is slightly affected by pronase digestion of intact cells (23). Both observations are consistent with component *a* being the anion channel, but go no way to prove it.

Major Glycoprotein

Isolation of the glycoprotein in a pure form is relatively simple; on extraction of ghosts with phenol, the glycoprotein partitions into the aqueous phase (24): in this sense it behaves like a nucleic acid. A useful refinement of this procedure has been developed (25). Some of the most extensive work on this molecule was carried out by Winzler and his colleagues (26). They showed that about two-thirds of its mass is carbohydrate, the rest being protein. With a molecular weight of around 30,000, it is only about 87 amino acid residues long, and carries approximately 100 sugar residues (27). Over a quarter of its amino acid residues are threonine or serine (27). A human red cell has about 7×10^5 copies of this glycoprotein (12), and this molecule accounts for 80 percent of the carbohydrate and more than 90 percent of the sialic acid (a negatively charged sugar) on the cell surface (26). The large net negative charge of human erythrocytes is attributable to this molecule. The carbohydrate region carries a variety of cell antigens, including the MN antigens, and acts as the influenza virus receptor (26).

Incubation of erythrocytes with trypsin releases most of the carbohydrate of the glycoprotein as a small number of glycopeptides. Because of the ease of isolation, glycopeptides from either cells or digested glycoprotein are a good source of material for studying carbohydrate sequences. Kathan and Winzler showed that the carbohydrate residues are attached to the polypeptide backbone not at one site, but at about ten different sites (27). The average size of the carbohydrate chains is therefore

small (ten residues or so). Winzler also showed that the carbohydrate is concentrated near the amino terminal region of the glycoprotein molecule and that another region, free of carbohydrate, is very insoluble. The glycoprotein can thus be viewed as a polypeptide, with carbohydrate clustered at one end and attached to the external surface of the membrane. Where is the rest of the molecule?

This can be studied by comparing those parts of the glycoprotein that are labeled from the cell exterior in intact cells with those labeled from both sides of the membrane in cell ghosts. This has been done with [³⁵S]formylmethionyl sulfone methyl phosphate as the labeling agent (12). The labeled glycoprotein was purified on SDS gels—an easy purification because the glycoprotein behaves quite differently from proteins on this fractionation system. Two-dimensional patterns of labeled peptides derived from the glycoprotein labeled in these two ways revealed that two peptides are heavily labeled in ghosts, but not at all in intact cells. These two labeled peptides therefore arise from a part of the glycoprotein on the cytoplasmic side of the membrane. This shows that the protein part of this glycoprotein extends across the membrane.

The simplest scheme for fitting this protein into the membrane may well be correct. This would place the NH₂-terminal region and associated carbohydrate on one (the outer) side of the membrane, followed by a region of α -helix to pass through the membrane, terminated at the COOH-terminal end of the molecule by a very hydrophilic tail. That part labeled in ghosts, but not in intact cells, should arise from this hydrophilic COOH-terminal tail. The portion of the molecule spanning the membrane would need to be somewhere around 25 residues in length (about 35 Å covered by 1.5 Å per residue in an α -helix) and would very probably have to be composed of hydrophobic residues. Preliminary evidence that a hydrophobic region does exist was found by Winzler (26); a hydrophobic sequence of 23 residues has recently been determined in this molecule (28).

Although no one knows what the physiological function of the glycoprotein is, it seems to me that it could serve two different functions that are not mutually exclusive. The first is obvious—it carries an enormous negative charge which serves to keep the erythrocytes apart from one another. The other possible function is that it

serves as a very primitive form of cell wall giving the erythrocyte rather more rigidity. The carbohydrate residues may not all be free in the aqueous phase, but, rather, may interact with one another by hydrogen bonding to form a lattice over the entire cell surface. Whether sugars are well suited for performing such a task we do not know at present. It seems to me that this glycoprotein is one of the most exciting molecules around to analyze by x-rays, not so much for its protein content, but to see how oligosaccharides might interact with one another.

Tektin A

It has been realized for some time that a substantial portion of the protein in erythrocyte ghosts has a high molecular weight and may have some function related to that of one of the contractile proteins (29). Until recently, the size of these molecules had not been properly appreciated. They are seen as two closely migrating bands on SDS gels, having apparent molecular weights of around 220,000 and 240,000. Neither protein is labeled in intact cells, but both are labeled in ghosts. Neither is degraded when proteases are added to intact cells, but both are sensitive when protease is added to ghosts. These, and other, data indicate that these proteins are associated with the inner surface of the cell membrane.

The most careful study of these two molecules has been made by Clarke (30) who calls them collectively Tektin A, and the two chains α and α' . These two chains are present in close to equimolar quantities. Tektin A can be eluted from ghosts in deionized water. The two chains have been purified together and only show one band when fractionated on gels which do not contain denaturing agents. Molecular weight determinations (in the absence of SDS) are complicated by the unusual property which Tektin A possesses of forming large filamentous aggregates when placed in dilute salt (0.1M KCl or 5 mM CaCl₂). However, cross-linking of Tektin A in dilute aqueous solution gives rise to covalent dimers, which suggests that the molecule itself has an $\alpha\alpha'$ constitution. Tektin A has another unusual property: it is soluble in 80 percent alcohol. These solubility properties suggest that it is probably rather hydrophobic and therefore that parts of it may lie within the lipid bilayer in the intact membrane.

Tektin A is a rod-shaped molecule having an axial ratio of about 45. In the electron microscope it is seen as an approximately 2000-Å rod, some 30 Å wide (30). If we take the protein content of a human erythrocyte ghost as about 7×10^{-13} gram, there should be some 2.2×10^5 molecules of Tektin A per cell. Given the dimensions of these rods, Tektin A could just about cover the entire inner surface (about 130 μm^2) of the membrane. Although this may be an overestimate of the area covered, there is no doubt that this molecule must play an important role.

It has been suggested several times that these proteins are "myosin-like." There is no good evidence for this; indeed, the solubility properties of Tektin A are the very reverse of those of myosin. Furthermore, the radioactive peptide patterns of α and α' obtained from human ghosts labeled with [³⁵S]-formylmethionyl sulfone methyl phosphate show no obvious resemblance to labeled rabbit skeletal myosin. These peptide patterns suggest, however, that α and α' may be related to one another.

Other Erythrocyte Membrane Proteins

The other main proteins found in the erythrocyte ghost have, in general, attracted less attention. None of them is labeled by membrane-impermeable reagents or degraded by proteases when these agents are added to intact cells. All are labeled or degraded when treated in ghosts. All of them are therefore associated with the inner surface of the membrane. Whether they are true membrane components—that is, whether they are associated with the membrane in the intact cell—is unknown. One of them has a molecular weight of about 40,000 and is a subunit of glyceraldehyde phosphate dehydrogenase (GPD) (31). Unlike most other membrane components, this GPD subunit can be eluted with dilute salt solutions from the ghost (32).

Freeze Fracture Microscopy

Proteins, such as component *a* and the glycoprotein, which extend across the bilayer might be seen when red cell membranes are examined in freeze fracture electron microscopy. In this process (33) the fracture plane passes through the middle of the lipid bilayer so that each half of the bilayer can later be viewed, as a replica, as one looks down

onto the inner surfaces of the membrane (34). Each fracture face of an erythrocyte ghost is littered with small (80 Å) particles, rather more of which are attached to the inner half of the bilayer than to the outer half (35). One might expect to find a "pit" in the smooth lipid surface for each particle which exists on the complementary surface. That these are not always found presumably reflects the flexibility of the hydrocarbon chains during fracture or replica deposition (carried out at about -100°C or higher).

The particles seen in cleaved erythrocyte membranes appear quite uniform in size and in this sense differ from those seen in other membranes. There are about 4200 particles per square micrometer and they appear to be randomly distributed (35). It seems most likely that these particles are component *a* (19); a protein of this size (10⁵ daltons) would have dimensions of about 50 by 50 by 100 Å. It has been suggested, with some indirect evidence, that the particles are the glycoproteins (36). This seems to me unlikely for several reasons. First, the particles are too big: that part of the glycoprotein which might reside within the bilayer is just too small in mass (less than 5000 daltons) to be 80 Å in diameter. Second, treatment of intact cells with proteolytic enzymes, which rapidly removes the bulk of the glycoprotein from the cell, does not affect the appearance of the particles (while component *a* is slightly modified to component *c*). This shows that the particles cannot be mainly the carbohydrate-bearing part of the glycoprotein. Finally, the number of particles (37) seen is about the number of copies of component *a* (6.2×10^5 compared to about 5×10^5 per cell). Unfortunately, this last argument is weakened because both component *a* and the glycoprotein are present in roughly equal numbers. Although I believe it is particles of component *a* that are seen in the fracture plane, it is just possible that the particles represent a complex of glycoprotein with component *a*.

This discussion brings out what seems to me to be one of the major technical problems facing interpretations of the freeze fracture technique: how to relate what is so beautifully seen in the microscope with molecules one knows about in the test tube. With other types of microscopy it is possible to tag structures with complexed antibodies of known specificity. Obviously, a new breakthrough is needed here.

Lipids

Almost one-half of the mass of plasma membranes of mammalian cells is lipid. It is conventional to place the lipids in three different categories: phospholipids (which comprise the major mass of the lipid), neutral lipids (such as cholesterol, which is usually found in large amounts in plasma membranes, but not intracellular membranes), and glycolipids. A few of the salient features of phospholipids are noted below; much more extensive and authoritative reviews exist to which I hope the reader will refer.

When a pure species of phospholipid is introduced into an aqueous environment, it spontaneously forms a multibilayered structure or smectic mesophase. This smectic mesophase exhibits a melting transition at a particular temperature (38) which depends critically on the nature of the fatty acid residues of the phospholipid; it is low for unsaturated phospholipids (about -10°C for dioleoyl lecithin) and much higher for saturated ones (61°C for distearoyl lecithin). Below the melting temperature the hydrocarbon chains are rigid, whereas above it they are free to move; their flexing motion is greatest at that end remote from the polar head group (39). Once the hydrocarbon chains have melted it may then become possible for individual phospholipid molecules to exchange with their neighbors, thereby leading to molecular lateral diffusion. Whether lateral diffusion of whole molecules occurs simultaneously with melting of the hydrocarbon chains is unknown. The rate at which lateral diffusion occurs was first studied by Kornberg and McConnell (40). Phospholipids move remarkably quickly, having an estimated neighbor exchange rate of somewhat less than 10^{-6} second (41). Such facile motion, which also exists in sarcoplasmic reticulum (42), is only slightly affected by addition of cholesterol to the bilayer (41).

A quite different type of molecular motion is of special interest for membranes. Kornberg and McConnell (43) found that the passage of a phospholipid molecule from one side of a bilayer to the other—a process they call “flip-flop”—is, by contrast, excessively slow. The half-life for a spin-labeled phosphatidylcholine to flip-flop was found to be about 6 hours at 30°C . In other words, lateral neighbor exchange occurs some 10^{10} times more rapidly than neighbor exchange across the

bilayer. It is unknown how the addition of cholesterol to a membrane would affect the rate of flip-flop (44).

The major phospholipids commonly found in mammalian cells are phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine. In general phosphatidylcholine and phosphatidylethanolamine are the most abundant, but this is far from being a rule. The most dramatic deviation is found in the erythrocytes of herbivores where phosphatidylcholine is essentially absent and seems to be replaced by sphingomyelin (45). As a general rule for erythrocytes from a wide variety of mammals, the proportion of phosphatidylcholine plus sphingomyelin is roughly constant (within the range 0.55 to 1.0) (46). These two species account then for about 60 percent of the total phospholipid. By contrast, the ratio of sphingomyelin to phosphatidylcholine varies from about 60 to 0.25. These arguments suggest that, in some loose sense, sphingomyelin and phosphatidylcholine can replace one another.

Elsewhere I have proposed that there is a profound reason for the apparent constancy of the proportions of choline-containing and amino-containing phospholipids in erythrocytes, and, by extension, in the plasma membranes of other mammalian cells (47). The interpretation is that the choline phospholipids constitute the outer half of the bilayer leaflet, and the amino phospholipids the inner, cytoplasmic, half of the bilayer. Several lines of evidence indicate that such an asymmetry exists. Perhaps the strongest general observation is that erythrocytes seem to have very few amino groups on their exterior surface. Two kinds of experiment indicate that this is so.

First, chemical agents which should react with amino groups on the cell exterior do not seem able to find them. This is true for ox erythrocytes or human erythrocytes, labeled with SITS (14, 23). In each case, intact cells were labeled to apparent saturation with this fluorescent agent; it was found that around 5×10^5 and 2×10^6 SITS combined with each ox or human cell, respectively. These figures are a long way from the number of amino phospholipids (about 10^8) which should exist on each cell surface, assuming a random distribution of them between the two halves of the bilayer. Furthermore, in both cases all the label appeared to be attached to protein and

none to lipid. Other, more extensive, experiments with FMMP to label amino groups lead to a similar conclusion, namely that reactive lipid amino groups are scarce on the surface of human erythrocytes but much more available in cell ghosts (48). A different type of chemical approach has been used by Bangham and his collaborators (49). They measured the mobility of erythrocytes in an electric field as a function of pH. In this way, they could titrate the surface and determine what type of chemical groupings exist there. Erythrocytes (from sheep) had a constant mobility between pH 4.5 and pH 11, indicating the absence of detectable surface amino groups (which should titrate around pH 10). In summary, chemical measurements fail to find the substantial amounts of amino phospholipids on the surface of erythrocytes which should be present if the bilayer had no compositional asymmetry.

The second line of evidence is provided by the effects of crude phospholipase A_2 preparations on erythrocytes. Phospholipase A_2 cleaves the fatty acyl ester bond on the 2 position of the glycerol moiety of any phospholipid, except for sphingomyelin, in which this linkage is effectively replaced by a peptide bond. Turner and his colleagues (45) discovered that sheep erythrocytes, which have no phosphatidylcholine, are resistant to the hemolytic action of the crude phospholipase A_2 of cobra venom. Erythrocytes from most other mammals are lysed by this enzyme. Turner *et al.* attributed this resistance to the replacement of phosphatidylcholine by sphingomyelin. The experiment suggests not only that sphingomyelin is present on the outer surface of sheep erythrocytes, but that amino phospholipids are not there in substantial amounts. This experiment has been repeated with a pure phospholipase A_2 on resealed ghosts derived from both sheep and human red blood cells. The results are consistent with the above experiments in which intact erythrocytes and a crude enzyme were used (50). A quite independent observation confirms the presence of phosphatidylcholine in the outer half of the bilayer of human erythrocytes (51). A crude enzyme from the sea snake, which does not lyse human erythrocytes, is nevertheless able to degrade some of the phosphatidylcholine when added to intact cells. When added to ghosts, the enzyme degrades not only phosphatidylcholine but also phospho-

tidylserine and phosphatidylethanolamine. This suggests that phosphatidylcholine, but neither of the amino phospholipids, faces out toward the external medium (52).

All this evidence suggests that the lipid bilayer of erythrocytes has compositional asymmetry: choline phospholipids on the external side, amino phospholipids facing the cytoplasm (53). The question arises whether this is also true of the plasma, and possibly other, membranes found in higher cells. It seems to me highly unlikely that such an asymmetry could arise just in the red blood cell. There must be a mechanism for generating the asymmetry, which is presumably inherent in the mechanism of biosynthesis of all membranes, and therefore it may be that the endoplasmic reticulum, Golgi apparatus as well as the plasma membrane, will all share this property. There is one hint that this may, indeed, be so. When a cell section is fixed only with osmium, the inner (cytoplasmic) side of each bilayer is much more readily seen than the outer half (54). Poststaining with uranyl acetate or fixation with permanganate enables both sides of the bilayer to be clearly seen. The interpretation of why osmium fixation only shows the inner surface clearly depends on what happens during this process. It is believed that, besides its reacting with double bonds in unsaturated lipids, osmium tetroxide also oxidizes amino groups. This would explain why phosphatidylethanolamine, but not phosphatidylcholine, binds osmium if both phospholipids contain only saturated fatty acids. The differential staining of the two sides of the bilayer by osmium *could* therefore reflect the differential content of amino phospholipids (55). It is therefore of considerable interest in this context to understand the chemistry of osmate fixation.

For unambiguously demonstrating a phospholipid compositional asymmetry, the most direct approach would be to measure the amount of each component on each side of the bilayer of a (red blood) cell. This could be done, in principle, in an experiment analogous to that of Deamer and Branton (56) in which a bilayer of sodium stearate, labeled on one side only, was cleaved by freeze fracture along the midplane of the bilayer. In this way they could separate the labeled from unlabeled sodium stearate. Returning to erythrocytes, if these were labeled *in vivo* with, for example, radioactive

choline, freeze fracture should cleave the radioactive choline residues from the inner half of the membrane bilayer. The techniques required for such an experiment make it extremely difficult to do at present.

Neutral Lipids

In a detailed x-ray analysis of the multilayered myelin membrane, Caspar and Kirschner (57) found that the electron density profile had a more pronounced shelf on one (the outer) side of the bilayer than the other (inner) side. This finding was interpreted as showing that there existed about twice as much cholesterol in the outer half of the bilayer as in the inner half. Whether this density shelf is, in fact, due to cholesterol and not to protein seems uncertain, although I believe that the overall interpretation of Caspar and Kirschner is probably correct. There is no other evidence on the location of cholesterol in plasma membranes.

Glycolipids

The observation that phospholipids only flip-flop across a bilayer with great reluctance (43), and the possibility that they may do so still less easily in a plasma membrane (44), raises the question of whether glycolipids can migrate from one side of a bilayer to the other. It seems to me that, like phospholipids, the movements of glycolipids in a membrane will be constrained to lateral diffusion alone. The situation which I surmise exists is that the glycolipid is located solely in the external half of the lipid bilayer. There is no firm evidence at present that this is so.

It is well known that cytoplasmic proteins are, as a general rule, never glycosylated, but that proteins secreted from mammalian cells (with minor exceptions) always are (58). It is also well established that all the carbohydrate of the major erythrocyte glycoprotein is external to the lipid bilayer (26). This is likely to be true of all membrane-bound carbohydrate attached to proteins, simply because of what is known of the process of glycosylation (59). Secreted proteins are synthesized in the rough endoplasmic reticulum and released into the cisternae where some glycosylation occurs; from there extensive glycosylation is

achieved as the protein passes through the Golgi apparatus before its eventual release from the cell. There is no reason to believe that the process of glycosylation either in the cisternae of the endoplasmic reticulum or in the Golgi apparatus distinguishes between membrane proteins or secreted proteins. Since the act of secretion is the synthesis of a protein which is separated, during synthesis, from the cytoplasmic pool of protein by the membrane of the endoplasmic reticulum, it is reasonable to imagine that all glycosylation occurs only on that, the noncytoplasmic, side of the membrane.

These arguments can be applied equally to glycosylation of lipids, and implies that glycolipids can only be found in the external half of a bilayer provided that no flip-flop occurs.

General Conclusions

We have, then, a picture of the erythrocyte membrane which is based on a lipid bilayer having compositional asymmetry in its phospholipid and glycolipid components. A major protein and glycoprotein are located in a fixed orientation across the membrane, and many more proteins are associated with the inner surface of the bilayer. We do not know whether, and to what extent, any of these latter proteins replace, or indeed penetrate, the inner half of the bilayer, although the inability to remove many of them by washing the ghosts with strong salt solutions implies that they probably are inserted into the bilayer. If this is so, there are two points to note.

First, the base composition of erythrocyte phospholipids shows that there are more external (choline) than internal (amino) phospholipids. The volume deficit within the inner half of the bilayer caused by this disparity may well be filled in by protein. That is, a substantial portion (maybe 30 percent) of the inner half of the bilayer may be protein. Alternatively, the suggested asymmetry of phospholipids may be less than complete; that is, some choline phospholipids may be located within the cytoplasmic half of the bilayer.

Second, the erythrocyte membrane also has compositional asymmetry within its hydrophobic phase. Phosphatidylcholine, sphingomyelin, and glycolipid all contain very few polyunsaturated fatty acid residues. By contrast, phosphatidylethanolamine and phospho-

tidylserine are rich in these components, particularly arachidonic acid (46). It is tempting to suggest that these polyunsaturated residues in the inner half of the bilayer provide a less ordered phase which may therefore be a better solvent for accommodating proteins as compared to the outer half of the bilayer.

The compositional asymmetry of a bilayer means that a membrane always has a polarity, even in the absence of any associated proteins. Cellular membranes can usually be given a gross polarity; one side is adjacent to the cytoplasm, the other side is remote from it. The generally accepted model for membrane fusion requires that this topological polarity be maintained. Thus, when secretory vesicles or membrane viruses fuse with the plasma membrane, or when two cells fuse, the cytoplasmic side of each membrane always remains on the cytoplasmic side of the fused membrane, whereas the external side is always kept external. The asymmetry developed here simply provides a molecular basis for defining each side of the bilayer.

The insertion into the bilayer of proteins which span the membrane provides another, more easily measured, polarity. I should like to dwell a few moments on how these proteins come to be in the membrane. There are two general classes of protein synthesized by a cell: cytoplasmic and secreted proteins. Cytoplasmic ribosomes synthesize cytoplasmic proteins such as ribosomal proteins, hemoglobin, or β -galactosidase. Membrane-bound ribosomes synthesize proteins for secretion and eventual export from the cell (59, 60). The decision of whether a protein is to be secreted or not—that is, on which class of ribosome it will be synthesized—must be genetically defined. The information for secretion presumably lies not in the nature of the protein product, but rather is encoded in the messenger RNA which codes for the protein. In other words, a messenger RNA must have information coded in it which determines the class of ribosome to which it can attach. Selection at this level determines the fate of the protein product. If the messenger RNA attaches to membrane-bound ribosomes, it seems probable that the secreted protein is extruded through the membrane as it is synthesized. This general scheme is consistent with recent studies of the specificity of protein synthesis by membrane-bound ribosomes (60, 61).

It is generally assumed that membrane proteins are synthesized by membrane-bound ribosomes, that they are a special class of secreted protein. This natural conclusion I believe is wrong. I have difficulties in seeing how membrane proteins can adopt their correct polarity in the membrane if they are extruded into it during synthesis. This problem is probably more acute for proteins which are only partially buried in the inner half of the bilayer. These proteins could find their correct location much more easily if they are synthesized in the cytoplasm, diffuse to the membrane, and insert themselves into it. The same process, I believe, is followed by proteins that extend across the bilayer. Synthesis by cytoplasmic ribosomes is followed by solution in the membrane. Whether a cytoplasmically synthesized protein remains as a soluble component, or partially dissolves in the inner surface of the bilayer, or dissolves in the membrane so that it traverses the bilayer is determined by the nature of the protein. For a transmembrane protein, the nature of the protein will ensure its correct polarity of insertion into the membrane; once inserted, glycosylation from the external side of the membrane will ensure a permanent abode for that protein across the membrane.

There is one piece of evidence which argues in favor of this hypothesis. The lactose (lac) operon of *Escherichia coli* has three genes that code for β -galactosidase, lactose permease, and thiogalactoside transacetylase. These three genes are transcribed and translated as a single messenger RNA (62). It is then very likely (especially in view of the polar nature of some z -gene mutants on the y -gene) that each of these genes is translated by the same class of ribosome. Since both the first and last genes on the messenger RNA (β -galactosidase and the transacetylase) are cytoplasmic proteins, the middle gene, coding for lactose permease (which probably spans the bacterial plasma membrane), must also be synthesized as a cytoplasmic protein. This general conclusion, that a protein which traverses the membrane has arrived in this position by diffusion from the cytoplasm, could explain why the major proteins exposed on the outer surface of the erythrocyte are so chemically unreactive; both the glycoprotein and component a seem to have few amino groups on their external surfaces. In the case of the glycoprotein, that part of it on the cell exterior is built from

rather neutral amino acids (almost 50 percent is accounted for by serine plus threonine (26). This NH_2 -terminal segment of the protein (without its carbohydrate) may spontaneously diffuse across the membrane.

To be as precise as possible, I should like to give an example of the implications of such arguments. The antigen receptor on B (bone marrow derived) lymphocytes is believed to be a 7S antibody molecule, built of two light and two heavy chains, located on the cell surface (63). After stimulation of the B lymphocyte by antigen, a series of cellular transformations occurs which results in an antibody-secreting plasma cell. It is widely believed that the antigenic specificity of these antibodies in these two states—surface bound or humoral—are identical. In other words, they are presumably coded by the same genes. In the surface-bound case, the F(ab) portion of the receptor resides on the exterior and contains the antigen-binding site (64). That end of the heavy chains remote from F(ab) presumably extends across the bilayer into the cytoplasmic region, perhaps to convey some message when antigen is bound (65). This is the simplest way of stably anchoring an antibody receptor in the cell membrane. Like other membrane proteins (66), this receptor can move laterally in the plane of the membrane (67). In the secreted state, the humoral antibody is free in solution, outside the cell. How can a protein exist in two such diverse states?

From my previous lines of argument it is possible to provide a variety of mechanisms for this, of which I regard the following as the most likely. The heavy chains of the receptor, which extend across the membrane, are cytoplasmically synthesized; the light chains, which are totally external, are secreted. The NH_2 -terminal region of the heavy chains is able to diffuse across the membrane where it is held fixed as the receptor either by combination with light chains or by glycosylation, or both. Upon induction and maturation of the B lymphocyte a switch has to occur. Not only the light chains but also the heavy chains are now secreted; the switch requires both heavy and light chain synthesis to occur on membrane-bound ribosomes. In other words, the maturation of a B lymphocyte to an antibody-producing cell requires a switch in the recognition of heavy chain messenger RNA by the two classes of ribosomes: from cyto-

plasmic in the B lymphocyte to membrane-bound in the plasma cell. No doubt the secreted antibody is glycosylated in places which, in the membrane-bound state, would have been inaccessible to the glycosylating enzymes.

Summary

The arrangement of lipids and some proteins in the erythrocyte membrane has been discussed. The conclusions from this are listed here as a set of general guidelines for the structure of membranes of higher organisms: some of these rules may be wrong. But at this stage it seems useful to sharpen our thoughts in this way and thereby focus attention on various specific points.

1) The basis of a membrane is a lipid bilayer with (i) choline phospholipids and glycolipids in the external half and (ii) amino (and possibly some choline) phospholipids in the cytoplasmic half. There is effectively no lipid exchange across the bilayer (unless enzymatically catalyzed) (68).

2) Some proteins extend across the bilayer. Where this is so, they will in general have carbohydrate on their surface remote from the cytoplasm. This carbohydrate may prevent the protein diffusing out of the membrane into the cytoplasm; it acts as a lock on the protein.

3) Just as lipids do not flip-flop, proteins do not rotate across the membrane. Lateral motion or rotation of lipids and proteins in the plane of the bilayer may be expected.

4) Most membrane protein is associated with the inner, cytoplasmic, surface of the membrane. Proteins are not usually associated exclusively with the outer half of the lipid bilayer.

5) Membrane proteins are a special class of cytoplasmic proteins, not of secreted proteins.

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