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The Plasma Membrane Consists of Two Polar–Nonpolar–Polar Leaflets

Interpretation of Some Data, Mainly from the Erythrocyte

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ABSTRACT

The implications of a double polar–nonpolar–polar leaflet construction of the plasma membrane are investigated. Experimental data from transmission electron microscopic and enzymologic characterization of plasma membranes are advantageously interpreted in these terms compared to interpretation in terms of lipid bilayer. X-ray diffraction and electron spin resonance studies do not differentiate between the present and previous models for the structure of plasma membranes but electron spin resonance data that fail to indicate a statistical distribution of spin labels also fail to support the fluid mosaic model for cell membranes. Results from experiments involving vectorial digestion and labelling of plasma membranes as well as freeze fracture electron microscopic data are compatible with the present model. The molecular composition of the human erythrocyte membrane is investigated whereby the band III protein and glycophorin are suggested to be the structural proteins of the outer leaflet and the spectrins those of the inner leaflet.

INTRODUCTION

The erythrocyte membrane is the most thoroughly investigated of biological membranes. Several approaches have been used in elucidating its structure, ranging from early estimations of lipid content (47) to recent X-ray photo-electron spectroscopy (69). The vast amount of information available on particularly the human erythrocyte membrane provides a basis for testing the recently developed concept that the plasma membrane consists of two polar–nonpolar–polar leaflets (15). This concept was originally developed from considerations of structural and functional similarities and dissimilarities between various biological membranes. In particular it required knowledge about the nucleotide metabolism associated with intact cells (3, 4, 5, 6, 29, 90) and elaboration (16) of the idea (58) that actin-containing filaments attached to the plasma membrane (52, 74) are analogous to the coupling factors (*cf.* 58) of mitochondria and chloroplasts. It is the aim of this work to examine data that are difficult to reconcile with the fluid mosaic model for the structure of cell membranes (95) and that tend to support the concept that such membranes consist of two parallel polar–nonpolar–polar leaflets. Primarily, data dealing with the human erythrocyte membrane will be discussed.

Some previous models for the structure of the human erythrocyte membrane

The lipid bilayer concept for the structure of biological membranes (see ref. 104 for a short review on membrane models) (Fig. 1) was originally developed from considerations of the surface area of intact erythrocytes compared with that occupied by the lipids extracted from erythrocytes when spread as a monolayer on an aqueous surface (47). Accord-

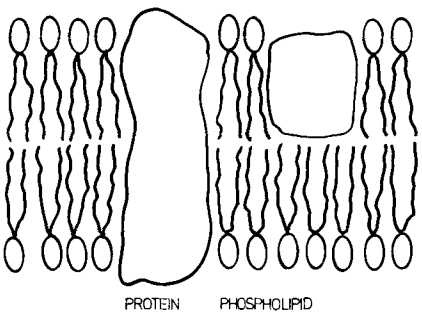

Fig. 1. Illustration of the fluid mosaic model for the structure of cell membranes according to which intramembranous particles revealed by freeze-fracturing represent globular proteins surrounded by unperturbed lipid bilayer (ref. 95).

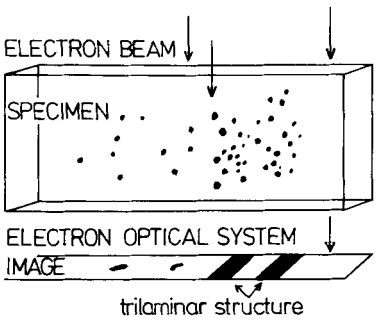

Fig. 2. Formation of a trilaminar image seen in the transmission electron microscope.

ing to this model (47, 88, 94, 95), a trilaminar appearance of the erythrocyte membrane under the transmission electron microscope is attributed to lack of affinity of stains to the nonpolar residues between polar residues of the bilayer (88). Fracture planes generated by the freeze fracture procedure for electron microscopic preparation are attributed to lack of cooperativity of van der Waals forces between methyl groups of contrapositioned lipids in the bilayer (25). Proteins spanning across two leaflets of the erythrocyte membrane (cf. 95) are claimed to be detected by the occurrence of intramembranous particles after freeze fracturing.

The stability of pure lipid bilayers (104) and the apparent lack of interdependence of protein and lipid conformation in biological membranes as interpreted from measurements of circular dichroism (94) have contributed significantly to the development of this concept (94, 95). However, lipid in bilayers is presumably equilibrated between membranes of various cellular systems by way of a small aqueous pool (104). It is therefore difficult to explain the fact that membranes from different organelles are characterized by different lipid composition (23, 70) and by characteristic enzymatic activities and proteins. This is because the fluid mosaic model for cell membranes implies that both lipid and protein move about, lacking close and specific contact in the planes of membranes (39, 95) that may in addition be capable of fusion (cf. 8). With this model it is also difficult to imagine any genetic control mechanism responsible for species-specific lipid composition of biological membranes (cf. 70, 120). Very complex mechanisms including restrained mobility of integral proteins imposed by specifically associated fibrous proteins have been developed (74) in order to overcome some of these difficulties.

The unit membrane hypothesis (88), according to which all biological membranes are represented by lipid bilayers with various amounts of loosely and peripherally associated protein, was partly based on electron microscopic observations of $KMnO_4$- or OsO_4-stained and dehydrated membranes. However, the thickness of the erythrocyte membrane, as determined by this methodology, is uncertain because of extensive losses of both protein and lipid when osmium and dehydration are used for electron microscopic preparation (67). In dehydrated preparations, cross-linked with glutaraldehyde and stained with low concentrations of osmium, thereby avoiding extensive losses of protein, no trilaminar structure can be seen and the membrane thickness is about 160 Å (67). Great variations of membrane thickness as measured by independent techniques (see Table I) indicate that the degree of hydration and the amounts of associated water-soluble proteins are important factors.

Table I. *The thickness of the (human) erythrocyte membrane determined by various methods*

For comparison, the thickness of a lipid bilayer may be 45–50 Å determined by X-ray diffraction (27, 104) and 45±5 Å determined by electron microscopy (72, 88). See ref. 71 for a more exhaustive constellation of data

Method	Thickness	Ref.
Birefringence	5 000 Å	71
Excluded volume	600 Å	44
Leptoscope (rabbit, rat steer)	160–220 Å	114
Argon ion etching combined with iron signals determined by electron spectroscopy	1 000 Å	69
Energy transfer	65–100 Å	82
Electron microscopy		
Glutaraldehyde and high concentrations of osmium tetroxide	70 Å	67
Glutaraldehyde and low concentrations of osmium-tetroxide	160 Å	67
Embedding in water-soluble polymers and staining with uranylacetate and lead citrate	70–110 Å	52, 81
The nonpolar core estimated from lipid content assuming even distribution in one surface layer	27 Å	33

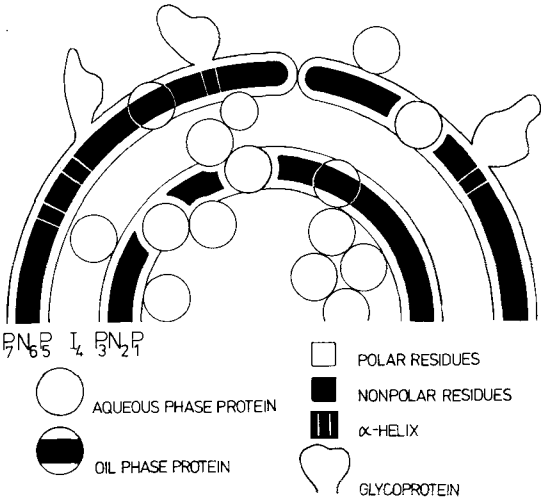

Fig. 3. The structure of a complete biological membrane according to ref. 15. The indexed capitals *P* (polar), *N* (nonpolar) and *I* (intramembranous) serve to denote the location of different components. For example, actin is a P_1 component of the plasma membrane, NDP-kinase and adenylate-kinase are presumably I_4 components of the mitochondrial and the plasma membranes (ref. 15).

Definition of a complete biological membrane

The nucleotide metabolism associated with the surface of many intact cells is similar to that associated with intact mitochondria (4, 6, 15, 29). Furthermore, actin-containing filaments, the subunit structure of which is reminiscent of that of the coupling factors of the inner mitochondrial membrane (58), are closely associated with the inner leaflet of the plasma membrane of many cells (52, 74). The similarity of the two mitochondrial membranes and the inner and outer leaflets of the plasma membrane is further emphasized by the asymmetric distribution of carbohydrates in both instances (10, 74, 75, 102).

In addition, covalently bound DNA is included as a marker for the inner leaflets in both these instances (cf. 15, 45, 50). By these criteria, the two mitochondrial membranes and the two leaflets of the plasma membrane may be designated as complete biological membranes (15, see Fig. 3). Since the outer leaflet of the plasma membrane contains identity markers and receptors of importance for cellular interactions in multicellular systems, there is reason to regard one complete biological membrane as a functional unit composed of one leaflet carrying endogenous information (DNA) and/or energy transducing activity and one leaflet mediating informational exchange with the surrounding medium (exogenous information). These functions may be more or less developed in particular instances. For example, endogenous information may be more important than the informational exchange function (exogenous information) in the complete biological membrane represented by the inner nuclear membrane + the outer nuclear membrane combined with the endoplasmatic membranes of animal cells.

Structure of a single leaflet

Nonpolar residues of amino acids and lipids tend to be shielded from hydrogen binding water-soluble compounds (104). According to this fundamental thermodynamic principle (the hydrophobic effect), a monolayer of phospholipids in contraposition to nonpolar amino acid residues of a peptide in a β-pleated sheet-configuration is stable, as illustrated in Fig. 4 A. This structure is maximally stabilized in an aqueous environment if every second amino acid residue of the peptide is polar. However, clusters of 15–17 nonpolar amino acids are stabilized in the form of an α-helix embedded in the nonpolar core (see Fig. 4B) because of the hydrophobic effect (104). The peptide may bind another layer of phospholipids after it has traversed the membrane, as illustrated in Fig. 4C. Similar arrangements of phospholipid, protein and other biological compounds may be imagined to exist throughout the three-dimensional membrane. This structure would be extremely stable owing to the contribution of cooperative nonpolar bonds, hydrogen bonds and salt bridges, as illustrated in

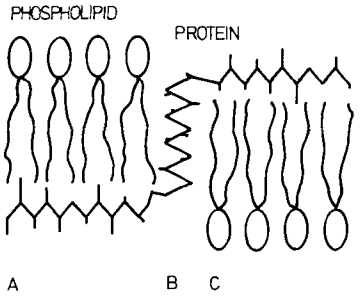

Fig. 4. Transsection of a monolayer of phospholipid and a peptide in β-pleated sheet configuration (A and C) and a peptide segment of 15–17 exclusively nonpolar amino acid residues stabilized in α-helix form in the nonpolar core (*B*).

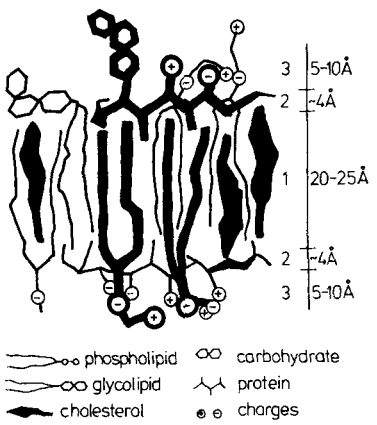

Fig. 5. Illustration of the possible contribution of lipid, protein and sugar to the structure of a single leaflet. Broad lines denote compounds located close to and thin lines far from the reader. This structure is composed of a nonpolar core (*1*), two planes of hydrogen bonding (*2*) and two planes of salt bridges (*3*). The binding of aqueous phase and oil phase proteins to this type of structure is illustrated in Fig. 3.

Fig. 5. The possible contribution of different compounds to these three types of binding is listed in Table II. The association of water-soluble and integral proteins as well as pore formation in one leaflet has been described previously (15).

Sugar residues are assumed (cf. 15) to profoundly influence a membrane on account of their nonpolar surfaces and their property to structurize water (cf. 35) thus presumably forming part of the planes of hydrogen bonding. The presence of sugar in biological membranes presumably make them more permeable to polar solutes by influencing the hydrophobic effect. This is illustrated tentatively by the decreased surface tension in lecithin monolayers brought about by sialic acid in combination with metal ions (26). Antigens and receptors residing in glycosyl residues would thus be shielded from detection until the membrane structure is disturbed by proteolytic or lipolytic cleavage. Thus treatment of fat cells with phospholipase C increases the insulin-binding capacity (24).

Some experimental data in support of this type of structure (see Fig. 5) may be accrued from the literature. For example the low extractability of camel erythrocyte phospholipid with ether/methanol (110) in contrast to that of human erythrocyte phospholipid of similar composition and of similar amount/surface area (30, 61) may suggest that protein is important in binding the phospholipids to the camel erythrocyte membrane which is characterized by a high protein to lipid ratio. If the thermodynamic principles denoted the hydrophobic effect are strictly applied to glycophorin (the sequenced main sialoglycoprotein of the human erythrocyte (62)) then the polar thr-74, ser-92 and under some conditions weakly acidic tyr-93 amino acid residues are preferably excluded from the nonpolar segment thought to extend through the lipid bilayer (cf. 104). The remaining segment, composed of 17 nonpolar amino acid residues and stabilized in an α-helix form due to the nonpolar environment (104) would

Table II. *Examples of atoms and groups in different biological compounds possibly contributing to the nonpolar core and the planes of hydrogen bonding and of salt-bridges in a biological membrane*

Compound	Nonpolar core	Plane of hydrogen bonding	Plane of salt-bridges
Cholesterol	$-CH_2-$, $-CH_3$, $-C=C-$	$-OH$	
Phospholipid	$-CH_2-$, $-CH_3$, $-C=C-$	$=O$, $-C=C-$, $-NH_2$ $-$inositol	$P-O^-$, $-$choline$^+$, $-$serine$^-$ $-NH_3^+$
Sphingolipid	$-CH_2-$, $-CH_3$, $-C=C-$	$=O$, $-OH$, $-NH$, $-C=C-$	$-P-O^-$, $-$choline$^+$
Glycolipid and carbohydrate	$-CH_2-$, $-CH_3$, $-C=C-$, nonpolar surfaces of carbohydrate	$=O$, $-OH$, $-NH$, $-C=C-$	$-$fucosate$^-$, $-$sialate$^-$
Peptides and protein	Nonpolar residues and atoms, α-helix	$=O$, $-NH$, polar residues, β-pleated sheet	Polar and ionic residues
	Interphase peptide of unknown configuration		
water	Polar "pockets"	$-OH$	$-OH$
metal ions			$+$, $++$

be 25.5 Å long (1.5 Å/residue, cf. 9) in good agreement with the postulated width of the nonpolar core according to the present model. The abundance of basic proteins in some biological membranes (e.g. myelin, ref. 34) is interesting because the positively charged residues of arginine and lysine are separated by 5 and 4 atoms respectively of similar size from a plane of hydrogen bonding (see Fig. 5) while a negatively charged phosphorous oxygen of phospholipid is separated from such a plane by 4–5 atoms, indicating the possibility of favourable conditions for electrostatic interactions between these residues. However, ionic solutes are probably important contributors to the planes of salt bridges (see Fig. 5). Ca^{++} ions have been reported to bind to the carboxyl groups of protein in the human erythrocyte membrane and these binding sites are shielded by amino groups (42, 43). In accordance with these findings, the carboxyl groups of glutamic and aspartic acid residues would be separated from a plane of hydrogen bonding by only 2 and 1 atom(s) respectively and shielded from the surrounding aqueous medium by the amino groups of phospholipid and protein (see Fig. 5 and Table II). Analytical freeze-cleaving has revealed that cholesterol may be enriched three-fold in the outer leaflet compared with the inner leaflet of the erythrocyte membrane (40). This finding is not compatible with the existence of a transmembranous nonpolar lipid core which would facilitate the rapid equilibrium of the nonpolar cholesterol molecule between the two leaflets in the absence of specific binding to protein and/or lipid.

Structural similarities between the present model and the bilayer model, of importance for experimental determination of physical parameters can be pointed out. These include the orientation of the axis of lipid perpendicular to the plane of the membrane and increased mobility of methyl groups in comparison with methylene groups. The latter are probably being more rigidly held by the planes of hydrogen bonding and fixed by adjacent nonpolar amino acid residues. If ions are excluded from the planes of hydrogen bonding and if cholesterol is present in the membrane (conferring on the lipids a preferred extended configuration, 18) then the region of comparatively low electron density would be 28–33 Å (see Fig. 5, the nonpolar amino acid residues contribute 2–5 Å). The presence of adsorbed ions in the planes of salt bridges would confer on these regions a higher electron density than that of the surrounding buffer. The planes of salt bridges containing electron-dense phosphorous and metal ions are probable reflection planes, which when detected by wide-angle X-ray analysis give rise to diffraction patterns. For example the Bragg spacing related to lipid in the bovine erythrocyte membrane as determined by wide-angle X-ray diffraction was 43 Å while that of extracted phospholipid was 51.5 Å (27). This discrepancy has led to the postulation of "tilted" phospholipid in the assumed lipid bilayer phase of that particular biological membrane (27). However, according to the present model for the plasma membrane the centres of the reflecting planes are between 28 and 53 Å (most probably 40–43 Å) apart (see Fig. 5) in better agreement with the 43 Å spacing reported from the bovine erythrocyte membrane. Thus, physical data which tend to support the lipid bilayer model, are also applicable to the present model for biological membranes.

However, biological membranes are regarded as delicate structures with optimized functions which have been acquired during the course of evolution. They are characterized by cooperative effects (98) and vectorial non-random motion in the plane of the membrane (7, 17), phenomena which are difficult to reconcile with the fluid mosaic model for cell membranes.

The present model is compatible with:

1. Translational rearrangements in the plane of a membrane coupled or not coupled to translational rearrangements in an adjacent membrane.

2. Vectorial movement of protein and lipid in "channels" (in the plane of a membrane) formed by the asymmetric apposition of protein, carbohydrate and lipid.

3. Rearrangements along an axis perpendicular to the plane of a membrane permitting transmembranous diffusion of macromolecules in their water-soluble form across one or two membranes (see figure 6 in ref. 15).

4. Adsorption of water-soluble enzymes, e.g. proteases and phospholipases with or without rearrangements and digestion of protein and lipid.

5. Shrinking of the membrane surface by folding, lipid bilayer formation and/or by exclusion of aqueous phase proteins.

6. Expansion of the membrane surface by integration of aqueous phase proteins in the plane of the membrane, β-pleated sheet formation or by unfolding of the entire membrane.

7. Cooperative effects mediated by conforma-

tional changes of lipid and structural protein and/or membrane-associated water (cf. 37) or by modified permeability properties of either of the two membranes.

8. Receptor–effector transducing mechanisms (e.g. the adenylcyclase system) across two membranes by (*a*) cooperative effects (see above), (*b*) proteins spatially linked in the intramembranous space (see Fig. 3), (*c*) penetration of the first messenger through one leaflet combined with allosteric regulation of enzymes in the other leaflet.

9. Dissociability of regulatory and catalytic subunits of hormone-sensitive enzyme complexes located in different membranes.

10. Specific or preferred interactions between protein and lipid, conferring on biological membranes individuality in each organelle and each cell line derived from different species. Thus, biological membranes may contain a significant amount of information and may be of semiconservative nature since lipid and proteins are partly complementary constituents of the membranes.

Fusion

The demonstration that fusion may occur (8), for instance between cells and extracellular vesicles, does not contradict the present hypothesis for the structure of plasma membranes. On the contrary, some experimental data from fusion experiments may be interpreted advantageously by assuming that the plasma membrane is two membranes. This is because the lipid vesicles may fuse either with the outer leaflet only, or with regions of the plasma membrane where the two leaflets are already fused. For example fusion with the outer leaflet would result in loading of the intramembranous space with compounds enclosed in the bilayer vesicles and leakage as has been reported (8) of these compounds through a "semiporous" outer leaflet. Fusion of human lymphocytes with mono-layered liposomes containing a fluorescent probe detectable only when diluted, resulted in fluorescence that was inhibited by 0.1% Triton X-100, thus suggesting either that the vesicles were adsorbed to the cell surface (116) or that the fluorescent probe was diluted in the entire cell (116) or in the intramembranous space after fusion of the vesicles with the outer leaflet.

Liposome–cell interactions involve adsorption of positively charged vesicles to the negatively charged cell surface (8, 65), endocytosis (8), and possible exchange of lipid between the cell surface and the liposomes (8). Complex interactions are presumably also involved in the fusion of human erythrocytes with lipid LP-X vesicles accumulating in serum during some hepato-biliary diseases since the fusion process (detected by enlarged surface area) does not occur in the absence of serum (111). The dilution of the intramembranous particles containing glycoprotein (see below) in these fusion experiments (111) may indicate that the intramembranous particles contain reserve material for forming membrane area. In agreement with this interpretation, antigens residing in the band 3 protein (see below) were detectable close to the intramembranous particles but not elsewhere (84), probably reflecting cryptic location at these other sites. Fusion of cells mediated by liposomes and virus membranes has sometimes been used as an argument for the occurrence of unperturbed lipid bilayer in cell membranes. However, if such were the case, the same argument would, contrary to what is generally observed, favour extensive fusion of cells in the absence of liposomes.

Fusion of intracellular vesicles with the plasma membrane (exocytosis) may in addition involve either displacement of the inner membrane or release of trapped compounds in the intramembranous space, followed by diffusion through the outer leaflet.

Present model applied to the human erythrocyte membrane

According to the present model, the two leaflets of the erythrocyte membrane which stain with osmium-tetroxide represent two nonpolar layers containing cholesterol and other lipids with carbon double bonds that induce precipitation of electron-dense osmium compounds (117). In addition, amino groups of protein (12) and polar heads of phospholipid (88) may contribute to osmium staining. Glutaraldehyde is believed to preserve a trilaminar appearance of the erythrocyte membrane of intact cells by cross-linking the proteins of the inner leaflet (cf. 67, 100).

The inner leaflet of human erythrocytes is preferentially visualized by glutaraldehyde fixation followed by embedding in water-soluble polymers and staining with uranyl-acetate and lead-citrate (51). The interleaflet space of the plasma membrane in such preparations where extensive extraction of lipid is avoided may be more than 70 Å (51, 80)

which is indicative of a polar character. This interpretation is supported by the facts that the interleaflet space in such preparations is usually more than 100 Å in tight junctions where the outer membrane leaflets of two adjacent cells adhere (80) and these preparations when stained with acidified silicotungstic acid reveal substantial gaps between tissue cells (81) probably corresponding to the outer leaflet containing carbohydrate and the intramembranous spaces.

The profound difference between the appearance of the plasma membrane and the intracellular membranes under the electron microscope is well-documented by several preparation techniques (51, 80, 96). It is also amply demonstrated by conventional preparation of vesicles derived from yeast cell plasma membranes and mitochondria (cf. 45). A thin electron-lucent rim in *one* leaflet of the plasma membrane can be seen in some electron microscopic images prepared by embedding in water-soluble polymers (51, 80, compare Fig. 5). Divalent cations may contribute to the electron density of both leaflets of the human erythrocyte membrane by association with negatively charged residues (42, 43).

The main contribution to a trilaminar image of the erythrocyte membrane would, however, be the additive electron density of components located at different depths of the transverse section (see Fig. 2). This superposition effect probably diminishes the importance of the specific affinity of stains to different chemical compounds and increases the importance of close spatial location of the components (cf. 15).

The fracture plane of the erythrocyte membrane is presumably created by shear forces, generated during freezing, between contracting (18, 104) nonpolar layers and the expanding aqueous phase. The outer leaflet is probably anchored in the extracellular ice by glycoprotein, while the inner leaflet is anchored in the intracellular ice by water-soluble protein (see Fig. 3) leading to predominant intramembranous splitting of the plasma membrane.

The lipid bilayer model may be included in this system as a special structure adoptable by a single leaflet or by fusion of two leaflets (see figure 4 in ref. 15). In addition one leaflet may be formed by proteins or by protein and lipid in close association. Perturbation of a delicate structure such as that illustrated in Fig. 5 could be expected to induce a lipid bilayer structure in one leaflet and consequent multiple fracture planes of the plasma membrane. This interpretation is particularly attractive in freeze-etch preparations where the cell surface may be identified (figure 10*a* and *c* in ref. 32).

The erythrocyte membrane is a delicate structure

The lipids of intact human erythrocytes are digested neither with pancreatic phospholipase A_2 (89), nor with phospholipase C from *Bacillus cereus* (89). However, lipids of the corresponding permeable ghosts are extensively digested by these and other lipolytic enzymes (46, 89, 112, 120). Hydrolysis of 20–48% of the total phospholipid of intact human erythrocytes by phospholipase A_2 from *Naja naja* or/and sphingomyelinase from *Staphylococcus aureus* results in ultrastructural alterations of both the inner and outer leaflets of the membrane (112). Similarly, digestion of intact rat erythrocytes with sphingomyelinase from *Staphylococcus aureus* and phospholipase C from *Bacillus cereus* under non-lytic conditions profoundly affects the appearance of both leaflets, leading to a significantly lower density of the intramembranous particles (46). Lipolytic enzymes from different sources digest phospholipids of intact erythrocytes from different species in a specific manner reflecting the protection afforded by adjacent molecules in the membrane (110, 112, 120). The degree of hydrolysis and the morphological effects of lipolytic enzymes upon erythrocytes influenced by the sequence of digestion (112), the intracellular ATP concentration (46), the presence or absence of extracellular albumin (49), and protecting effects of non-digestible lipid and protein (110, 112, 120). The complexity of these interactions is difficult to reconcile with the fluid mosaic model for cell membranes, according to which most of the erythrocyte surface is formed by unperturbed lipid bilayer.

Molecular composition of the two leaflets of the human erythrocyte membrane

The major proteins of the human erythrocyte membrane have been characterized by their electrophoretic migration in SDS-polyacrylamide gels (102) whereby mainly 7 protein-staining (bands I–VII) and 3 carbohydrate-staining (the sialoglycoprotein, glycophorin) bands have been distinguished. By various techniques, including protease digestion and the labelling of intact erythrocytes and resealed ghosts with radioactive markers, only band III and

glycophorin of the main proteins have been shown to be exposed at the outer surface (102). These proteins, both of which are hydrophobically associated with lipid, constitute about 30% (102) of the total protein mass and the remaining 70% are not to a similar extent detectable from the outside (102).

Some of these remaining 70% detectable in leaky ghosts (102) or inside-out vesicles are susceptible to crosslinking agents (100) and may form a continuum in the intact membrane (12, 102). Two of them, the band I and II proteins, constitute about 30% of the total protein mass (102) and are jointly designated spectrin. It has been estimated that the amount of spectrin is sufficient to cover the inside of the erythrocyte (12). Immunological methods have established that spectrin is located on the cytoplasmic side of the membrane (76). The solubility of these proteins in 80% ethanol (20) and their capability for both polar and nonpolar interactions with phospholipid (53) suggest that they may be associated with lipid *in situ*. They can be extracted from human ghosts by incubation with cholate (22), EDTA (87) or neutral and alkaline buffers of low ionic strength (87). These procedures lead to vesiculation of ghosts, solubilization of up to 90% of the membrane proteins (87) and/or alteration of the trilaminar structure obtained by osmium-staining the pelleted material obtained from high-speed centrifugation (22, 87). Extraction with water under gentle stirring seems to specifically elute spectrin leaving a residue of atypical trilaminar membranes loosely associated with osmiophilic droplets which may represent phospholipid (87). In this connection it is noteworthy that phospholipid–glycoprotein aggregates (115) as well as proteins (36) and lipid bilayers (72) may give rise to trilaminar images in the electron microscope. The fact that osmium staining and dehydration leads to significant losses of protein and lipid (67) is also pertinent. Spectrin is an elongated (fibrous) protein in aqueous solution (20) but fibrous networks have never been observed by freeze-etching to cover the inside of erythrocytes or ghosts (108) although other fibrous proteins are detectable with this technique (106). Part of the spectrin is firmly associated with camel erythrocyte ghosts and is experimentally defined as an integral (not extractable by EDTA or slight modifications of ionic strength) protein in that system (30). Camel erythrocyte ghosts contain five times more integral protein per unit surface area than do human ghosts and a higher density of intramembranous particles which are also larger (30). Extraction of spectrin from camel erythrocyte ghosts leads to conformational changes in the membrane as evidenced by decreased surface area and increased density observed in phase contrast microscope (85). Vesiculation of human ghosts by incubation with protamine is accompanied by the appearance of multiple fracture planes some of which form in the inner leaflet (the author's interpretation of figure 10*a* and *c* in ref. 32) and the shedding of protein-free lipid vesicles (32). The conclusion that lipid bilayer is formed from structures such as that illustrated in Fig. 5 during vesiculation is (contrary to the fluid mosaic model) compatible with the *increased* fluidity of lipids (and decreased mobility of protein) in the ghost membrane as measured by electron spin resonance (1) under conditions which lead to vesiculation (cf. 32).

Other evidence (cf. 101, 102) indicates that the band V (actin-like polypeptide) and the band VI (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) proteins are components of the inner leaflet. The band IV and VII proteins are however more difficult to locate. The band I, II, V, VI and VII proteins can be extracted from ghosts by incubation with cholate (22) indicating that this procedure preferentially solubilizes the inner leaflet. After extraction, the remaining thin (22) trilaminar structure is predominantly composed of the band III and IV proteins, the sialoglycoproteins and some lipid (22). This cholate-extracted cholesterol and lipid-depleted structure should be expected to exhibit staining properties markedly different from those of the native membrane. Incubation in 8 M urea also seems to elute preferably the proteins of the inner leaflet leaving mainly the band III protein associated with about 50% of the lipid (54). On the other hand, Triton X-100 and Tween 20 preferably solubilize the proteins of the outer leaflet (22, 59). The contours of the ghosts can be seen with the aid of the phase contrast microscope during elution with cholate or Triton X-100 (22). Ghost-like structures can also be seen after these procedures by electron-microscopic examination (22). These structures are globular after extraction with Triton X-100 and trilaminar after extraction with cholate (22).

These data justify the suggestion that spectrin is the structural (48) protein of the inner leaflet while the band III protein and glycophorin are

the structural proteins of the outer leaflet of the human erythrocyte membrane. (The hydrophobic segment of glycophorin is only about 25.5 Å (see above) which is not sufficient for spanning two leaflets. See also page 176 for an evaluation of experimental data concerning the localization of proteins in native membranes.) If spectrin is not associated with lipid *in situ*, then a highly ordered network of hydrogen bonds and salt bridges must be assumed to exist in the inner leaflet in order to account for the low permeability of the erythrocyte membrane towards various polar solutes. Since spectrin may be partly extracted from human ghosts (full extraction requiring sonication) with apparently maintained structure (31), it is reasonable to assume that other proteins as well contribute to the formation of the inner leaflet.

The extracellular activity of GAPDH and other glycolytic enzymes which is firmly associated with intact cells (3, 6, 90) may reflect the "semiporous" properties of the outer leaflet. It is notable in this connection that phosphate added to Hela cells and erythrocytes may first enter the 1,3-diphosphoglycerate and ATP pools (77) indicating that phosphate transport is actually mediated by GAPDH, which presumably holds a transmembranous position in the inner leaflet. The outer leaflet of the human erythrocyte membrane may be regarded as the diffusion barrier inferred from studies of glucose and galactose transport (86), while the carrier mechanism for these solutes is situated in the inner leaflet and is destroyed concomitantly with the proteolytic cleavage of spectrin by proteolytic enzymes included in resealed ghosts (66). This is also supported by the finding that GAPDH (and aldolase) most of which is bound to the *inner* (101) surface of the ghost membrane is more available to its substrate than are a number of cytoplasmic enzymes (e.g. lactate dehydrogenase and pyruvate kinase) under various conditions (28) indicating that this enzyme (and aldolase) constitute or is located *exterior* to a diffusion barrier (presumably the inner leaflet). These interpretations are corroborated by the demonstration that the enzymatic properties of GAPDH are not significantly different in the membrane-associated and solubilized states (91).

The Na^+- and K^+-stimulated ATPase is presumably located in the inner leaflet (cf. 63). Ouabain and potassium are regarded as being capable of influencing the activity of this enzyme by binding to the outer surface of the inner leaflet after passage through the outer leaflet.

The intramembranous particles

Conditions which labilize the spectrin (the band I and II proteins) tend to facilitate aggregation of the intramembranous particles (31). Cross-linking of spectrin with glutaraldehyde inhibits particle aggregation (31) (as well as solubilization of these proteins by sonication in the absence of divalent metal ions (31)). These and other findings (84) suggest that spectrin is involved in creating or forming the intramembranous particles seen by freeze fracture (31, 84). There is also much evidence correlating the intramembranous particles with glycophorin, the main sialoglycoprotein of the human erythrocyte membrane (107). However, recently it was shown that mutant erythrocytes which lack this protein still contain intramembranous particles (13). Ferritin-conjugated antibodies directed against antigens residing in glycophorin as well as in the band III protein bind to the surface of human erythrocytes in a pattern that is reminiscent of the pattern formed by the intramembranous particles (84, 107). Thus spectrin, glycophorin, the band III protein and other proteins and lipid (60) as well, may be involved in forming the intramembranous particles which are tetrameric or heterogeneous when visualized by rotatory replication (64).

Several difficulties attend the correlation of morphological observations on freeze-fractured specimens with other parameters. These include variations due to the choice of freeze fracture equipment and methodology (60), the proportion of cross fractures to tangential fractures (60) and altered fracture planes (99). The findings that fixation (79) cooling before freezing (118) and the presence of dimethylsulphoxide or glycerol (57) affect the particle frequency of various biological membranes indicate that particles are impermanent structures. They do not necessarily represent proteins of high molecular weight since they are found in protease-digested membranes lacking high molecular weight proteins (119) and in artificial membranes containing hydrophobic peptides (92).

The fluid mosaic model for cell membranes predicts that the density of the intramembranous particles representing intramembranous protein (94, 95) should increase when lipid but not protein is removed (21, 60) from the membrane by digestion

with phospholipase C. This is because the smooth fracture planes are considered to represent the nonpolar region of the membrane built up of lipid bilayer (95) in which the integral proteins are freely (39) diffusible (95) and appear after freeze-etching as intramembranous particles. Contrary to this prediction, a decreased density of the particles following digestion with phospholipase C was reported (60). Ghosts digested maximally with phospholipase C very rarely split tangentially (60). When they do, smooth fracture faces are occasionally (60) but not always (112) produced. If the membrane-associated particles of ghosts are permanent *intramembranous* structures, it is unlikely that their higher density would counteract the tangential splitting of the plasma membrane, since aggreagated particles have been visualized repeatedly (84, 107).

Exogenously applied proteins of high molecular weight (e.g. actin, MW: $n \times 43\,000$; ferritin, MW: 450 000; the tetramer of phytohemagglutinin, MW: 126 000; the tetramer of Concanavalin A, MW: 110 000) bound to the cell surface may be visualized by the freeze fracture procedure (83, 84, 106, 107). If it is assumed that endogenous globular and fibrous peripheral proteins of high molecular weight (e.g. actin, MW: $n \times 43\,000$; spectrin, MW: 200 000–240 000, and the tetramer of GAPDH, MW: 140 000) are also visualized by this procedure, then they must be confined to the intramembranous particles. This is because both the intracellular and extracellular surfaces are comparatively smooth (see ref. 83). These considerations are compatible with the view that proteins experimentally defined as peripheral proteins (94, 95) partly form the inner leaflet of intact erythrocytes and minimally perturbed ghosts. The significance of water-soluble proteins in forming membrane area is also adduced from the observation that extensively washed and resealed ghosts are smaller than slightly washed and resealed ghosts (55).

It follows from these considerations that the outer leaflet cannot be a lipid bilayer, since such structures are cleaved smoothly by freeze-fracture, and no fracture planes are seen within the outer leaflet under ordinary conditions.

Current status of previous reports

Proteins spanning two leaflets of the plasma membrane (95) were included in the original version of the present model for biological membranes with some reservations (15). However, other features of the model such as the postulated complex interactions between various residues forming the planes of hydrogen bonding and salt bridges and also the transmembranous diffusion of water-soluble protein must be taken into account. Such phenomena may partly compensate for the apparent vectorial labelling and digestion of intact cells, resealed ghosts, resealed inside-out and right side-out vesicles and leaky ghosts (cf. 102). The interdependency of protein and lipid conformations in erythrocyte membranes is documented by electron-spin resonance studies (93, 103, 113). The complex pattern of digestion of intact erythrocytes with lipolytic enzymes (110, 112, 120) is tentatively explained by polar interactions between protein and lipid. In this connection is may be added that iodination of *intact* mitochondria with the lactoperoxidase method results in labelling of *one* component apparently related to the *inner* membrane (11) and the same procedure applied to intact erythrocytes may cause labelling of hemoglobin (109). Thus, the existence of proteins spanning two polar–nonpolar–polar leaflets with those properties required by the present model for the structure of plasma membranes and other biological membranes is not corroborated by the available evidence.

The globular structures preserved by some electronmicroscopic preparatory techniques (67, 68, 96, 97) may be interpreted as foldings of one leaflet around globular water-soluble structures (15). Other arrangements are equally possible, the characterization of which depends on further definement of the preparatory techniques (97).

General considerations

By analogy with the inhibitory effect of proteolytic enzymes on glucose and galactose carrier function in erythrocytes (66), the function of giant axons is similarly disturbed by proteolytic action on the inner leaflet of the axolemma (105). A thermodynamic analysis of nerve cell depolarization across two membranous leaflets separated by a polar space has been presented and found to describe satisfactorily the experimental data (56). The interpretation of data from nerve cell axons is complicated by the occurrence of serial membranes in addition to the two leaflets of the plasma membrane. However, according to the double leaflet model for biological membranes only the inner leaflet is involved in energy transducing activity (15). Therefore, the periaxonal space deduced from experimental devia-

tions from theoretically expected depolarization currents (2) may partly represent the intramembranous space. The possibility of a sodium gradient primarily across the inner leaflet (cf. 15) is an attractive explanation why the generalizing of the concept of sodium-dependent transport of certain metabolites across cellular membranes (cf. 19) fails experimentally (73).

The main criticism that can be directed against the lipid bilayer concept for biological membranes involves the fact that no other structure which explains the experimental data has seriously been considered. The controlled references in the experiments have of necessity been lipid bilayers or protein in aqeous media. For example the fact that fracture planes are generated in the nonpolar core of lipid bilayer (25) seems to suggest that noncooperative hydrophobic interactions are always responsible for the fracture planes in biological membranes. Estimation of the amount of a α-helix, β-structure and random coil in biological membranes rests on the use of water-soluble reference peptides without considerations of additional interactions possibly occurring in biological membranes. Interpretation of X-ray diffraction data involves a comparison with electronmicroscopic pictures that are considered to represent bilayers. Interpretation of electron spin resonance data sometimes rests on the assumption that the lipid is statistically distributed in the native membrane which is assumed to be a lipid bilayer. However, electron spin resonance studies should be interpreted with care, since a statistical distribution of spin labels and lipid is not to be expected *a priori* (93, 113).

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