Analytical Techniques

The discovery and development of new tools for the analysis and identification of archeological materials is proceeding in many laboratories throughout the world, and only the barest outline of the work such tools are performing for archeology will be given here (*25*). Neutron-activation analysis, based upon nuclear transmutation caused by bombardment in a nuclear reactor, may be used for widely varying analyses—on blood and soil, surveying the Mohole, analyzing the suface of the moon, or for the nondestructive analysis of ancient pottery and metals. The Brookhaven National Laboratory has used neutron-activation analysis to demonstrate, with pottery from Italy and from Central America, that a detailed analysis of elements contained in the clays makes it possible to determine the source of the materials and perhaps the region of manufacture. For example, the fine orange ware found at Piedras Negras in the lowlands of Guatemala has been proved to have been fabricated from deposits located in the highlands.

The Research Laboratory for Archaeology and the History of Art at Oxford has reported on a number of techniques currently under investigation, which, like the neutron-activation method, can be used for both qualitative and quantitative studies of archeological materials directed at tracing the origin of manufacture, trade routes, the understanding of ancient technology, and the detection of fakes. These techniques include x-ray fluorescence, electric-beam x-ray-scanning microanalysis, beta-ray backscatter meters, and optical-emission spectrometry. The essential point, however, is that these are archeological tools recently derived, for the most part, from postwar atomic-nuclear development. And it is their large number and rapid rate of improvement which indicate the probable future impact upon archeological studies.

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23. Surveys at this site were conducted under the sponsorship of the Canadian Department of Northern Affairs and Natural Resources as a part of a training program for students in archeology.

24. We are indebted to M. J. Aitken, Research Laboratory for Archaeology and the History of Art, Oxford University, for these measurements.
25. Most of these are reported in *Archaeometry* **1** to **7** (1958–1964).
26. We thank the NSF for support of our thermoluminescence, C^{14} known age dating, and part of our instrument programs.

Structure of Biological Membranes

The unit membrane theory is reevaluated in light of the data now available.

Edward D. Korn

Membrane biochemistry occupies a central position in modern biology, second in importance, perhaps, only to biochemical genetics. Replication and organization are the significant differences between living and nonliving catalytic systems, and cellular organization is a function of membranes. In spite of the fact that many of the major activities of cells occur in, on, or through membranes, very little is known about their structure or the mechanisms of membrane-associated reactions.

One hypothesis for the structure of membranes that has been generally accepted is the concept of the unit membrane as proposed by Robertson (*1*). This theory is a skillful interpretation of electron microscopic and x-ray diffraction data in terms of the ingenious paucimolecular model of membrane structure deduced by Danielli and Davson (*2*) from permeability, surface tension, and electrical conductivity measurements.

The unit membrane theory has two aspects. First, there is one basic structure to which all membranes, or most portions of all membranes, of all cells of all species conform. Second, this structure consists of a bimolecular leaflet of phospholipids whose nonpolar portions, mainly fatty acyl chains, are inwardly oriented perpendicular to the plane of the membrane. The polar moieties of the phospholipids comprise the external surface of the bimolecular leaflet and are covered by a layer of protein and, perhaps, some carbohydrate. It is understood that the com-

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position of the phospholipids may vary and that appreciable concentrations of cholesterol and minor amounts of other neutral lipids may be present. The nature of the protein is not specified but again it is understood that, although there may be a "structural" protein, much of the protein undoubtedly consists of enzymes that will be different for different membranes. It is important to remember the proposed universality of the unit membrane, that its structural basis is the bimolecular leaflet of phospholipid held together mainly by van der Waals forces among the hydrocarbon chains, and that polar bonds are supposed to bind the proteins to the phospholipids. I shall show that: (i) the unit membrane theory is not proven by the data that led to its formulation, (ii) recent data are difficult to rationalize on the basis of the unit membrane concept, (iii) the unit membrane theory is difficult to reconcile with current concepts of molecular biology particularly with regard to genetic control of membrane structure and physiological and biochemical regulation of membrane function, and (iv) the data necessary to test critically the correctness of the theory are still not available. It is the major purpose of this discussion to stimulate experiments that will test the validity of the theory.

Unit Membrane Theory and Myelin

Three different types of evidence are adduced in support of the unit membrane theory: chemical, electron microscopic, and x-ray diffraction data (*1, 3, 4*). Similar conclusions were reached much earlier from polarized light microscopy but these data will not be discussed (*5*). It is important in assessing the strength of the argument to realize that the universality of the concept is dependent entirely on electron microscopic observations, that chemical data are available for only a very few membranes and have been utilized only with regard to the erythrocyte ghost, and that the x-ray diffraction data are limited to model systems and to one biological membrane —the myelin sheath.

Myelin is a multilayered structure surrounding single axons of peripheral nerves. It appears to be an internal proliferation of the plasma membrane of the Schwann cell that surrounds axons of unmyelinated as well as myelinated nerves. In unmyelinated nerves only one layer of the Schwann cell plasma membrane encases the axon (*6*) while myelinated axons are surrounded by multiple layers derived from the plasma membrane of a single cell (*7*). The highly ordered and repeating pattern of myelin has allowed analysis by x-ray diffraction.

The original low-angle diffraction patterns obtained by Schmitt (*8*) showed the presence of a radially oriented repeating unit with spacings of about 180 to 185 Å for mammalian peripheral nerve. These data were interpreted in terms of repeating units containing *two* bimolecular leaflets of phospholipid approximately 140 Å thick (one bimolecular leaflet formed by lipids extracted from nerve was found to be 60 to 70 Å thick) interspersed with a protein layer of approximately 25 Å. Later analyses by Finean (*9*) showed that under certain conditions the size of the repeating unit could be halved. He, therefore, proposed a model in which the smallest repeating unit consisted of one bimolecular leaflet that was 50 to 55 Å thick with a protein layer that was 30 Å thick. Alternate layers of the repeating unit were thought to differ in some constituent, the "difference factor," which reconciled the two models. The x-ray data do not define the type of bond between the protein and lipid, nor do they indicate the orientation of the lipid within the bimolecular layer.

The next conceptual stage was the correlation of the x-ray data with the electron microscopic image of myelin. During the early 1950's, Fernandez-Moran and Sjöstrand had independently obtained high resolution micrographs of myelin of nerves fixed in OsO_4 (*10*). The main feature of these micrographs was a series of dense lines about 25 Å thick, separated by about 125 Å, surrounding the axon. Occasionally an intraperiod line was seen which was much less dense, thinner, and more irregular than the major dense line. When nerve is fixed with $KMnO_4$, however, an intraperiod line of similar thickness to the major dense line is always seen and after prolonged fixation (7 to 12 hours) the two lines become indistinguishable (*11, 12*).

During an incisive investigation, Fernandez-Moran and Finean (*13*) performed x-ray diffraction analyses on nerve at several stages during preparation of the tissue for electron microscopy, thus correlating the information from these two different techniques. The 180-Å repeating unit of myelin in fresh nerve was observed to shrink about 20 Å during fixation in aqueous OsO_4, and to reach a value of 130 to 140 Å in the completely dehydrated and methacrylate-embedded specimen. Finally, when viewed in the electron microscope, the major dense bands were spaced at intervals of approximately 120 Å. The additional 10- to 20-Å shrinkage was presumably caused by the environment within the microscope. A significant observation, not often emphasized, was the marked difference in the x-ray patterns of fresh nerve and the wet OsO_4-fixed material. In addition to the shrinkage already noted, the distribution of x-ray diffracting power was markedly shifted. The intensity of the 160- to 180-Å spacing increased and the intensity of the 80- to 90-Å spacing decreased. This uneven distribution agreed with the electron micrographs of nerve fixed in OsO_4 which showed major dense lines and only faint intraperiod lines. In contrast, fixation with $KMnO_4$ resulted in relatively little change in the x-ray diffraction pattern. The relative intensities of the primary and secondary reflections remained essentially equal, as they were in fresh nerve, in agreement with the equivalent densities of the major and intraperiod lines in the micrographs.

While these data support the opinion that the repeating units of x-ray-diffraction and electron microscopy are related, they give no information on the orientation of lipid layers or the nature of the presumed lipid-protein bonds. Fernandez-Moran and Finean (*13*) pointed out that it is uncertain what the dense areas of electron micrographs represent chemically. They suggested that a light atom such as manganese (atomic weight 55) might not contribute significantly to the electron density. The only effect of $KMnO_4$ fixation may be to stabilize the structure. The observed electron densities would be due to the elements originally present in the myelin (thus the good agreement between x-ray data and micrographs of permanganate-fixed material). Osmium tetroxide (atomic weight of osmium, 190) might "re-organize the lipid or protein components" and have a dominant staining effect. Robertson (*1*) has also suggested that "it is probable that an important part of the observed densities (in electron micrographs) are due to the underlying structures." This point has been elaborated by Ornstein (*14*) who has questioned whether osmium is directly responsible for the

enhanced electron density of material fixed in OsO_4. He has suggested that fixation might cause a redistribution of the compounds that are normally present in the tissue, and would result in local concentrations that are sufficiently electron-dense to cause images. Just such a redistribution of cell material might explain the change in the x-ray diffraction patterns after fixation with OsO_4. However, Merriam has offered indirect evidence that OsO_4 is directly responsible for at least some of the electron density of fixed tissue (*15*).

Electron Microscopic Observations

Derivation of the unit membrane theory from the proposed structure of myelin depends entirely on the facts that myelin appears to originate from the plasma membrane of the Schwann cell and that electron micrographs of this membrane resemble those of other biological membranes. Fixed in $KMnO_4$, the Schwann cell plasma membrane exhibits the typical triple-layered structure which consists of two dense lines, each less than 25 Å, separated by a much less opaque region. The entire structure measures 75 Å across. In favorable sections, this membrane appears to be continuous with myelin. It is as if the plasma membrane had wrapped around the nerve axon, followed by a fusion of the apposed inner (cytoplasmic) surfaces of adjacent layers of membrane and the apposed external surfaces of the membrane in the next layer (*12*). The fused inner surfaces of the membranes form what appear as the heavy, dense bands in micrographs of nerve fixed in OsO_4, and the fused outer surfaces form the less dense intraperiod bands. Thus one opaque band in micrographs of myelin is thought to be the product of condensation of *two* opaque portions of the triple-layered plasma membrane. The unit of myelin that consists of two major dense lines and an intraperiod line would be derived from six dense lines of Schwann cell plasma membrane which represent two complete triple-layered structures and halves of two other areas of triple-layered membrane. The plasma membrane of the Schwann cell would correspond to one-half of the 180-Å repeating unit of myelin. The plasma membrane would then consist of one bimolecular leaflet of lipid coated on the inner and outer surfaces with a layer of protein. This is the unit membrane.

Myelin swells when hydrated. Electron micrographs show that the major dense lines are still intact, but that there are separations between the fused outer surfaces of the membranes (*1*). Thus the outer surface of the original membrane may be more hydrophilic than the inner surface, perhaps because of the presence of polysaccharide which may, therefore, be the "difference factor" proposed by Finean from the x-ray data.

Although impressive, this argument is not fully convincing. The repeating unit of myelin is supposed to be the product of the fusion of two plasma membranes, but the distance between two dense bands in micrographs of myelin (120 Å) is about 30 Å less than twice the width of the plasma membrane of the Schwann cell. Similarly, the major dense and intraperiod lines of myelin are no wider than one of the opaque lines of the Schwann cell plasma membrane even though one of the former is supposed to be derived from two of the latter. Finally, the apparent contiguity of the Schwann cell plasma membrane and myelin has led to the assumption that their structures must be the same. As I will show later, myelin is chemically, metabolically, and functionally very different from all other membranes for which data are available. If the plasma membrane of the Schwann cell resembles other plasma membranes, then it cannot be very much like myelin. If the Schwann cell plasma membrane resembles myelin, which I think unlikely, then it cannot be very much like other membranes. In either case it seems unreasonable, from the available evidence, to generalize from the structure of myelin to other membranes.

The electron microscopic data for other plasma membranes have recently been summarized (*16*). In general, triple-layered structures are observed after fixation in $KMnO_4$ and often, but not always, after fixation in OsO_4. It is, of course, well known that other cellular membranes, the endoplasmic reticulum (cytomembranes), the outer, inner, and cristae membranes of mitochondria, chloroplast membranes, the two membranes of the nuclear envelope, and the membranes of bacterial protoplasts and spheroplasts are also revealed as triple-layered structures in electron micrographs. In general, membranes are thought to be about 75 Å wide. The variations in dimensions of membranes, however, have perhaps not been suitably emphasized. The overall widths of triple-layered plasma membranes appear to vary from about 50 Å to perhaps 130 Å (*16*). How much of this variation is due to differences in the methods of preparation and how much to fundamental differences in structure is not clear. At the very least, these variations indicate the difficulty of interpreting micrographs in terms of molecular structure.

Perhaps the clearest electron micrographic indication of differences among membranes was obtained by Sjöstrand (*17*) who compared adjacent membranes in single cell sections of mouse kidney and pancreas fixed with OsO_4 and $KMnO_4$. The thinnest membranes (mitochondrial and α-cytomembranes) were 50 Å to 60 Å, and the thickest membranes (plasma and zymogen granules) were 90 Å to 100 Å. Furthermore, Sjöstrand (*18*) observed globular subunits in one of the opaque layers of mitochondrial membranes and smooth endoplasmic reticulum, and an asymmetry in the electron opacity of the dense lines in the plasma membrane. Subunits, or cross-linkages bridging the gap between the two opaque bands of the triple-layered structure, have been observed by Robertson (*19*) who later reinterpreted them as an electron optical artifact derived from a mosaic pattern in the plane of one or both surfaces of the triple layer (*20*). In several instances, hexagonal mosaic patterns have been seen on the surfaces of plasma membranes (*21*). The strongest evidence for membrane subunits is contained in a recent paper by Blasie, Dewey, Blaurock and Worthington (*22*). Outer segment membranes of frog retina were isolated and were oriented in ultracentrifugal pellets. Electron microscopic surface views of negatively stained membranes and low-angle x-ray diffraction patterns from unfixed, unstained pellets showed a square array of spherical particles. The unit cell size was about 70 Å and the particles had a nonpolar core about 40 Å in diameter.

Model Systems

The interpretation of x-ray data and electron micrographs of biological membranes has been strongly influenced by the belief that the bimolecular leaflet is the most probable form, if not the only form, that is assumed by phospholipid micelles. It is

neither possible nor necessary to review all the work on model systems. It is sufficient to recall that tubular forms often appear when crude or purified phospholipids are mixed with water. These can be studied by x-ray diffraction and electron microscopic techniques. In general, the x-ray diffraction data have been interpreted to show the presence of radially oriented, lamellar structures made up of bimolecular leaflets. The spacings vary with the water content and the lipid composition. Typical results are those of Bear *et al.* (*23*) who found a spacing of 43.5 Å for the phospholipids lecithin and cephalin. These structures can be "fixed," sometimes with $KMnO_4$, but usually only by OsO_4, and viewed in the electron microscope. Perhaps the best results were obtained by Stoeckenius (*24*) who found a striated pattern of dark bands (approximately 18 Å wide) 40 Å apart. It has never been pointed out that the close agreement between x-ray and electron microscopic data for model systems differs significantly from the observations on myelin where the spacings on electron micrographs are less than 60 percent of those determined by x-ray diffraction.

Recent work has thrown some doubt on the logic of correlating biological membranes with these simple structures. Luzzati and Husson (*25*), in an important study, demonstrated that a phospholipid mixture from human brain can exist in at least two different liquid crystalline forms. One of these is the familiar lamellar arrangement of biomolecular leaflets. The other is an hexagonal array of circular cylinders in which each cylinder is thought to consist of a channel of water surrounded by the polar moieties of the phospholipids. The hydrocarbon portions of the phospholipids occupy the spaces between cylinders. Perhaps significantly, the hexagonal arrangement was favored by temperatures between 30° and 40°C, and by higher concentrations of phospholipids. Stoeckenius (*26*) has obtained micrographs that show a hexagonal array of dots by fixing phospholipid micelles at 37°C with OsO_4 vapor.

More recently, Lucy and Glauert (*27*) obtained electron micrographs of mixtures of lecithin and cholesterol negatively stained with phosphotungstic acid. A variety of forms—lamellar, tubular, hexagonal, and helical—were seen, many of which could be explained only as aggregates of lipid micelles about 40 Å in diameter. They further suggested that even the lamellar arrangements might be organized layers of micelles rather than bimolecular leaflets. In cross section these might well appear as triple-layered structures in which the two dark bands represent parallel layers of micelles. The individual spherical micelles might not be seen because of the relative thickness of even a "thin" section. The suggestion of layers of micelles is reminiscent of the arrays of micelles that have been seen on negatively stained surfaces of plasma membranes (*21*) and which are almost identical to the surface view of retinal outer segment membranes (*22*).

All these systems are probably poor models for biological membranes since they contain no protein and even their lipid compositions do not resemble those of natural membranes. But the models do emphasize that phospholipids, and especially mixtures of phospholipids and cholesterol, often exist as micelles, not bimolecular leaflets, and that even very small changes in the environment are critical. Stoeckenius (*26*), for example, has suggested that fixation with aqueous OsO_4 might change the arrangement of molecules in phospholipid structures, including membranes, and Lucy and Glauert (*27*) have observed that OsO_4 converted helical arrangements of lipid micelles into stacked disks.

Chemistry of Electron Microscopy

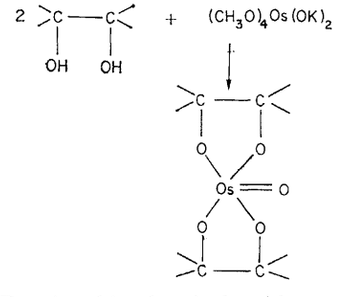
Much of the evidence for the unit membrane structure depends on the interpretation of electron micrographs in molecular terms. To do this, it is necessary to know what atoms are responsible for the electron microscopic image. If these atoms were introduced during fixation or "staining," it is essential to know with what functional groups of what molecules the visualized atoms have reacted and to prove that molecular orientations have not been altered by the procedures employed in microscopy. Electron microscopy may have its own uncertainty principle. However, too little is known about any of these points to permit the deduction of molecular structure from micrographs of membranes.

It has already been mentioned that both Robertson (*1*) and Finean (*13*) have questioned whether the manganese atom is responsible for any of the electron opacity in micrographs of $KMnO_4$-fixed cells. If it is not, there is no way to interpret the dense lines in micrographs of membranes fixed with $KMnO_4$ in molecular terms. This is a serious deficiency because it is in such preparations that the triple-layered structure is most reproducibly and distinctly seen. There appears to be only one study in which cells were chemically studied during fixation with $KMnO_4$. Korn and Weisman (*28*) found the lipids of amoebas to be essentially unaffected by fixation with 1 percent $KMnO_4$ for 1 hour at 0°C. All the neutral lipids and about half of the phospholipids were extracted from the amoebas during dehydration in ethanol.

Claims have been made, however, that OsO_4 is a marker for the polar end of phospholipids. This is the main, if not the only, experimental support for proposing that the bimolecular leaflet of lipid of a unit membrane is oriented with the polar groups outward, that is, the dense bands of the triple-layered image represent osmium bound to the polar portions of phospholipids and protein. I have evaluated the evidence for this conclusion in detail elsewhere (*29*).

Any discussion of the chemistry of osmium tetroxide fixation must start with the work of Criegee (*30*). In two monumental papers he demonstrated that OsO_4 reacts stoichiometrically with olefins to form a stable osmic acid ester of the glycol derived from the olefin by oxidation:

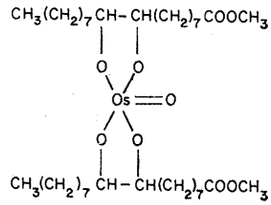
This product could be hydrolyzed to free glycol, but only under reasonably strong conditions. In a similar reaction starting from a glycol and tetramethyl dipotassium osmate, a dimer could be synthesized:



Despite this chemical evidence Stoeckenius (*31*) interpreted the similarity of electron micrographs of uranyl linolenate before and after exposure to vapors of OsO_4 and of potassium linol-

enate after fixation with vapors of OsO_4 as an indication of binding of the osmium to the end of the polyolefinic acid that contained the uranyl ion, the carboxylate group. No chemical evidence for any reaction between OsO_4 and uranyl or potassium linolenate was provided, nor was a mechanism proposed to explain the affinity of osmium for the uranyl or potassium carboxylate. Riemersma and Booij (*32*) studied the reaction between OsO_4 and phosphatidyl choline in aqueous *t*-butanol. They found that the amount of OsO_4 bound to the lecithin was exactly equivalent to the number of double bonds in the molecule, but suggested, on the basis of qualitative color tests on silicic acid chromatograms of the products, that, although OsO_4 initially attacked the olefinic group, the osmium subsequently migrated from the hydrocarbon end of the molecule to the polar end where it was bound to the quaternary amino group of choline. Finally, Stoeckenius (*33*) studied the products of reaction of OsO_4 with oleic acid in chloroform at room temperature for 24 hours. Based on infrared spectra of the crude reaction products in Potassium bromide pellets, he also concluded that the osmium had come off the double bond where it had reacted initially, leaving behind a glycol, and had formed an osmium carboxylate. None of these investigators propose a mechanism whereby osmium tetroxide, having attacked the double bond to form an osmic acid ester, could then migrate to the other end of the molecule. It should also be noted that, whereas Stoeckenius suggested that the osmium is bound to the anionic carboxyl group, Riemersma proposed that the osmium is bound to the cationic quaternary nitrogen. Finally, none of these reactions has been carried out under the conditions that are used for the fixation of biological material.

In a recent group of papers (*28, 29, 34*) I have shown that when methyl oleate is reacted with a 2 percent solution of OsO_4 in water for 1 hour at 0°C, it is quantitatively converted to bis(methyl-9,10-dihydroxystearate)osmate with probably the following structure:



This stable product was isolated by thin-layer chromatography and characterized by gas-liquid chromatography, infrared, nuclear magnetic resonance, and visible spectra, molecular weight determination, and elemental analysis. It is identical by all criteria to the product synthesized by reaction of methyl-9,10-dihydroxystearate with tetramethyl dipotassium osmate. More recently, the analogous product with a free carboxyl group has been synthesized from oleic acid and OsO_4, and from 9,10-dihydroxystearic acid and tetramethyl dipotassium osmate. Also, osmic acid diesters were found to be the product of the reaction between aqueous OsO_4 and a dioctadecenoyl lecithin. Furthermore, bis(methyl-9,10-dihydroxystearate)osmate has been isolated from amoebas following fixation with OsO_4 in Millonig's buffer. There thus seems to be strong support for the conclusion that OsO_4 reacts with the olefinic groups in lipids to form the stable osmic acid esters of glycols and, therefore, that osmium is covalently bound to the hydrocarbon portions of lipids in membranes fixed with OsO_4. In the model reactions, however, an approximately equal amount of osmium was recovered as uncharacterized products, presumably lower oxides. The origin of this product is still not clear, but it is possible that osmium other than that bound at the hydrocarbon portions of fatty acids may be deposited in tissues after fixation by OsO_4. Which osmium, if either, is responsible for the image is not known.

The final consideration is whether structures that contain lipids are unchanged after fixation with OsO_4. It would seem likely that replacement of a double bond by a glycol osmate ester would have some effect on the configuration of the lipid. Furthermore, since the product of the reaction of OsO_4 and the monounsaturated oleic acid is a dimer, one could imagine that the polyunsaturated fatty acids of tissues might form polymeric structures linked by diesters of osmic acid. This possibility was suggested by Wigglesworth (*35*) and Baker (*36*). Polymerization might explain why OsO_4 is a good fixative (it would cross-link lipids in the way that glutaraldehyde probably cross-links proteins), but it is not reassuring from the point of view of the maintenance of the original molecular arrangements.

I conclude from the chemical data that the dense lines in membranes fixed with osmium tetroxide reveal nothing about the molecular orientation of the phospholipid, in the original membrane. This conclusion is reinforced by the fact that triple-layered membranes were seen in osmium-fixed mitochondria from which all the lipid had previously been removed by extraction with acetone (*37*), and that *Escherichia coli* B (*38*) can be shown to have a triple-layered membrane despite the fact that this organism contains essentially no unsaturated fatty acids. If lipids are not necessary to reveal the triple-layered structure then its image cannot be used to deduce the arrangement of lipids in the membrane. Finally, the only available direct evidence is incompatible with the proposal that membrane proteins and phospholipids are linked through polar bonds. A "structural" protein, that accounts for about 50 percent of the total protein, has been isolated from bovine heart mitochondria (*39*). This insoluble protein binds phospholipids, irrespective of their charge, through nonpolar, hydrophobic bonds (*40*).

Chemical Composition of Membranes

The unit membrane theory imposes at least one specific restriction on the chemical composition of membranes. The lipid content must be sufficient to cover the surface area of the membrane with a bimolecular leaflet. The protein content might be more variable depending on the thickness of the layer covering the surfaces of the lipid. Although data are rapidly accumulating on the composition of the lipids and phospholipids of cells and some membrane systems, in only one case are the data available to calculate the relation of protein and lipid to surface area.

When the unit membrane theory was proposed, the only available data were those of Gorter and Grendel (*41*) on the erythrocyte. These investigators extracted whole erythrocytes with acetone, determined the surface area occupied by the lipids on a Langmuir trough, and compared this value to the surface area of erythrocytes calculated from dried smears. In two experiments with human erythrocytes, the ratio of surface area occupied by lipid to surface area of the cell was 1.9 and 2.0, in good agreement with the proposed bimolecular leaflet. These data, however, depended on several unproved assumptions: that all the lipid of the erythrocyte is in the plas-

Table 1. Protein and lipid composition of animal and bacterial membranes.

Origin of membrane	Molar ratio*			Area ratio (protein/lipid)†
	Amino acid	Phospholipid	Cholesterol	
Myelin (*46*)	264	111	75	0.43
Erythrocyte (*47*)	500	31	31	2.5
Bacillus licheniformis (*48*)	610	31	0	4.8
Micrococcus lysodeikticus (*48*)	524	29	0	4.3
Bacillus megaterium (*49*)	520	23	0	5.4
Streptococcus faecalis (*50*)	441	31	0	3.4
Mycoplasma laidlawii (*51*)	442	25.2	2.3	4.1

* Data are calculated from the percentage compositions given in the references indicated, using the appropriate molecular weights. † The approximate area occupied by a monomolecular film assuming an average amino acid occupies 17 Å^2 (*49*), a phospholipid molecule 70 Å^2 (*4, 23*), and a cholesterol molecule 38 Å^2 (*4*).

ma membrane; that this lipid is totally extracted by acetone and recovered without loss; that the surface area measurement in a Langmuir trough is a valid measure of the area occupied by lipid in a membrane; and that the surface area of red cells is unchanged in dried smears. Retrospectively, it is clear that not all these assumptions were valid. Although it is probably true that essentially all of the lipid of the erythrocyte is contained in the plasma membrane (erythrocyte ghost) (*42*), the lipid is not completely extracted by acetone. There was a compensating error, however, of greater magnitude in the measurement of the surface area of the red cell. From contemporary data of Ways and Hanahan (*43*), one can calculate that one human erythrocyte contains 2.7×10^8 molecules of phospholipid (4.5×10^{-16} mole) and 18.6×10^7 molecules of cholesterol (3.1×10^{-16} mole). If one assumes that one phospholipid molecule occupies 70 Å^2 of surface area and one cholesterol molecule occupies 38 Å^2 (*4, 23*), then the lipid of one erythrocyte would cover a surface area of 260×10^8 Å^2, or 260 μ^2. Since a modern value (*44*) for the surface area of a human erythrocyte is 167 μ^2, the ratio of surface area of lipid to surface area of plasma membrane is 1.56. There is some evidence (*45*) that a mixture of phospholipid and cholesterol in the above ratio would in fact occupy only 90 percent of the surface area occupied by the molecules separately, and so a more accurate value might be 1.40. In any case, the number approximates the required value of 2, but is it close enough? Unfortunately, data exist for no other membrane that permit a similar calculation.

There are, however, data for several plasma membrane preparations that allow the calculation of the ratio of protein to lipid and, with certain assumptions, the ratio of surface area occupied by protein to surface area occupied by lipid. These data cannot, however, be compared to the surface area of the membrane. One might expect that if all membranes were very similar, their protein to lipid ratios might be similar. Also, under the simplest, but not necessarily correct, assumption for the structure of the unit membrane, it might be anticipated that the surface area occupied by a monomolecular film of the protein and lipid components would be equal.

It is immediately apparent from the data in Table 1, that the protein-lipid ratios of membranes vary. Calculating the data for the best described system, myelin, one finds [using a value of 17 Å^2 for the area of one amino acid residue of a protein in either an α- or β-conformation (*47*)] that the protein content is sufficient to cover only 43 percent of the area occupied by the lipid. Therefore, much of the lipid surface area in myelin cannot be covered with a layer of protein, if the lipid is in a bimolecular leaflet. A similar calculation for the erythrocyte ghost demonstrates that there is sufficient protein to give a monomolecular film with 2.5 times the area of the lipid. An approximately similar value can be calculated from the incomplete data for liver cell membranes (*52*). Most bacterial membranes seem to have enough protein to cover five times the area occupied by the lipid. There is, of course, no proof that extraneous protein does not contaminate some of these membrane preparations nor is it necessary to assume that the hypothetical bimolecular leaflet is covered by protein in the form of a monomolecular layer. Nevertheless, these data are the best, indeed the only available, and it must be concluded at this stage that differences do exist among membranes and that myelin is very different, by this criterion, from all other membranes.

A closer look at the chemical data from the point of view of the composition of the lipids of various membranes will be useful. A few examples from the burgeoning literature (*53*) will suffice (Table 2). The major phospholipids of myelin are the cerebrosides, but no other animal or microbial membrane contains any of these compounds. Sphingomyelin is an important constituent of the erythrocyte plasma membrane and of myelin, but is probably not present in mitochondrial membranes, possibly not present in all microsomal membranes, and certainly absent from microbial membranes. Bacterial membranes contain no steroid and this is probably also true for at least one amoeba (*57*). The lipid composition of bacterial membranes is very limited (Table 2). For example, *Azotobacter agilis* and *Escherichia coli* have only one phospholipid, phosphatidyl ethanolamine.

Fatty acid compositions vary even more widely. Phosphatidyl ethanolamine and phosphatidyl serine of myelin (*58*) contain about 10 percent tetra-, penta-, and hexa-unsaturated C_{20} and C_{22} fatty acids. The rest of their fatty acids are oleate, and oleate and stearate, respectively. The same phospholipids of erythrocytes (*43*) contain about 40 percent polyunsaturated C_{20} and C_{22} fatty acids. Myelin phosphatidyl choline (*58*) contains essentially no polyunsaturated or $C_{20\text{-}22}$ fatty acids. Its major fatty acids are palmitate and oleate. The major constituent of erythrocyte phosphatidyl choline is linoleate (*43*). From 25 to 80 percent of the fatty acids of myelin cerebrosides are α-hydroxy fatty acids (*58*). These fatty acids are unique to brain.

The greatest contrast appears when one looks at the fatty acids of bacteria. Gram-negative bacteria contain almost exclusively saturated and monounsaturated C_{16} and C_{18} fatty acids (*59, 60*). No polyunsaturated fatty acids are found and a new type of saturated fatty acid frequently appears, the cyclopropane fatty acids (for example, lactobacillic acid, an 18-carbon acid with a methylene bridge between positions 11 and 12). *Escherichia coli* strain B may contain no unsaturated fatty acids (*61*). Membranes of Gram-positive bacteria have been found to contain mainly branched chain C_{15} and C_{17} fatty acids and in two cases,

Table 2. Lipid composition of animal and bacterial membranes.

	Myelin (46)	Erythrocyte (43)	Mitochondria (53)	Microsome (53, 54)		*Azotobacter agilis* (55)	*Escherichia coli* (55)	*Agrobacterium tumefaciens*	*Bacillus megaterium* (56)
Cholesterol	25	25	5	6	*	0	0	0	0
Phosphatidyl ethanolamine	14	20	28	17	18	100	100	90	45
Phosphatidyl serine	7	11	0	0	9	0	0	0	0
Phosphatidyl choline	11	23	48	64	48	0	0	10	0
Phosphatidyl inositol	0	2	8	11	6	0	0	0	0
Phosphatidyl glycerol	0	0	1	2	0	0	0	0	45
Cardiolipin	0	0	11	0	2	0	0	0	0
Sphingomyelin	6	18	0	0	9	0	0	0	0
Cerebroside	21	0	0	0	0	0	0	0	0
Cerebroside sulfate	4	0	0	0	0	0	0	0	0
Ceramide	1	0	0	0	0	0	0	0	0
Lysyl phosphatidyl glycerol	0	0	0	0	0	0	0	0	10
Unknown or other	12	2	0	0	0	0	0	0	0

* Not analyzed.

Lineola longa and *Micrococcus lysodeikticus*, no unsaturated fatty acids are present (*60*).

The major conclusion to be drawn from all these chemical data is that a wide spectrum of membrane composition exists. At one extreme is myelin which has the lowest protein content, a high percentage of unique phospholipids, and a high percentage of unique α-hydroxy fatty acids and saturated fatty acids. At the other end of the spectrum are the bacterial membranes which contain a high ratio of protein to lipid (ten times that of myelin), no steroids, and often only one phospholipid. It may be possible for membranes with these differences to exist in the same unit membrane structure. But it is worth considering that the chemical differences among membranes are certainly more definitive, and are probably more significant, than the generally similar appearance of membranes in electron micrographs.

Biological Considerations

At the present stage of knowledge, comments relating membrane structure to membrane function are entirely speculative. In any consideration of membrane structure, however, it is important to remember the many diversified tasks with which membranes are concerned. A partial list would include protein biosynthesis; energy transduction; transport of amino acids, sugars, and other small molecules; flow of water; maintenance of specific ion balances; pinocytosis and phagocytosis; a variety of reactions of intermediary metabolism; and controlled growth and division. All these processes involve proteins as enzymes or "carriers." It is difficult to imagine that membranes carrying out these exceedingly intricate and dynamic processes would all have the same structure, especially a structure shared by myelin. Myelin is metabolically inert and, as far as it is known, has only one function—that of an electrical insulator. This role of myelin might be best served by a structure that consists of repeating layers of lipids. I find it difficult to envisage how the many activities of other membranes could be performed by a structure whose fundamental properties are largely dictated by the physical-chemical characteristics of its lipid components. I would prefer to think of the protein component of membranes as the functional unit and that, as with modern architectural theory, form follows function rather than function being restricted within the confines of structural limitations.

Similar conclusions can be reached by considering possible mechanisms of the biosynthesis of membranes. The unit membrane concept includes, and is to a significant extent derived from, the belief that under the appropriate conditions phospholipids form bimolecular leaflets. According to this view the bimolecular leaflet provides more than the forces that maintain the integrity of the membrane. The fundamental step in membrane synthesis would be the formation of a bimolecular leaflet of lipids to which the metabolically functional proteins subsequently attach. This process necessitates either that the arrangement of lipids in the membrane, which later specifies the disposition of proteins, is a random functioning of lipid composition or that the cell specifically controls the orderly synthesis and organization of lipids independent of protein. Neither alternative is consistent with contemporary molecular biology. I find it more reasonable to suppose that the first step in membrane biosynthesis is the synthesis of the protein components as directed by genetic information. Lipoproteins would then be synthesized by the binding of lipids in a manner dictated by the amino acid sequences of the proteins. Serum lipoproteins seem to be synthesized in just this way (*62*). Finally, membranes would be formed by aggregation of the lipoproteins. Although the possibility exists, there is no reason to suppose that lipoprotein subunits would rearrange into a bimolecular leaflet. The globular subunits that are seen in electron micrographs of negatively stained membranes, and sometimes in cross sections of positively stained membranes, might be the lipoprotein molecules.

If membranes consist of lipoprotein subunits it should be possible to recover them from membranes. Razin and his colleagues (*51*) have reported that the surface membrane of the pleuropneumonia-like organism *Mycoplasma laidlawii* dissolves in a solution of sodium dodecylsulfate to give ultracentrifugally homogeneous subunits that, upon removal of detergent, reaggregate into membrane-like material, although 50 percent of the original protein was lost. Similarly, Salton and Netschey (*63*) have dissolved membranes from *Micrococcus lysodeikticus* and *Sarcina lutea* with detergent solutions and obtained fractions which were

homogeneous in the ultracentrifuge. Bovine myelin has been made completely soluble by detergents to an electrophoretically homogeneous lipoprotein (*64*). Rat myelin (*65*) can be made completely soluble by lysolecithin to homogeneous subunits with a molecular weight of about 500,000. Similarly, erythrocyte membranes (*66*) have been dissolved in detergents to give a subunit that was reported to be homogeneous as judged by electrophoresis and sedimentation in an ultracentrifuge. Membranes of *Halobacterium cutirubrum*, a bacterium that grows only in solutions of sodium chloride that are at least 3*M*, dissolve in distilled water (*67*). The ability to recover lipoproteins from membranes does not, of course, disprove the unit membrane theory, but it is a necessary consequence of a structure composed of lipoprotein subunits.

Conclusions

The combined x-ray diffraction and electron microscopic examination of myelin has provided reasonable, but not conclusive, support for its structure as a basically bimolecular leaflet of phospholipid that is partially interspersed with protein. But there is very little basis for extending this concept to biological membranes in general. There is no adequate experimental support for the specific orientation of phospholipids as proposed in the unit membrane theory or for the proposed polar nature of protein-lipid bonds, even in myelin. Membranes differ widely in chemical composition, metabolism, function, enzymatic composition, and even in their electron microscopic image. The only similarity is their general resemblance in electron micrographs, but, until more is known about the chemistry of electron microscopy, this evidence cannot be interpreted with confidence. One positive conclusion to which I have come is that much more chemical evidence must, and can, be obtained. Techniques for the isolation of membranes are improving and protein and lipid chemistry are now highly refined arts. Quantitative analysis of many different membranes is possible and the data can be related in some instances, notably bacterial plasma membranes, to calculations of surface area. Chemical and physical changes induced in membranes of widely different lipid composition by the preparatory procedures of electron microscopy can be determined directly and correlated with the electron microscopic image. Model systems can be assembled whose compositions closely resemble those of biological membranes. Membranes can be dissociated into subunits whose properties can be studied. In particular, x-ray diffraction analysis and electron microscopy by negative staining of reaggregates of lipoproteins isolated from membranes would be very informative. Perhaps most important, the problem of membrane structure must be considered in relation to the problems of membrane function and membrane biosynthesis.

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68. I thank Drs. R. W. Berliner, C. L. Greenblatt, R. W. Hendler, and R. A. Weisman for their helpful criticism.