REVIEW

CURRENT MODELS FOR THE STRUCTURE OF BIOLOGICAL MEMBRANES

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I. INTRODUCTION

During the last 100 years permeability studies had led to the conclusion that there exists on the surface of cells a thin membrane which controls the exchange of water and solutes between the cell and its environment. The accumulated indirect evidence had been so compelling that the direct

visualization of the membrane in the electron microscope and its isolation through cell fractionation from a variety of different cells were taken merely as a gratifying confirmation of a well-established theory. However, the simultaneous discovery, by the same techniques, of a multitude of intracytoplasmic membranes which separated intracellular compartments and had apparently partly similar functions was totally unexpected. It emphasized the important role that membranes play in the structure and function of cells and made the study of membranes a fundamental topic in modern biology.

Unfortunately, these discoveries so far have produced few new insights into the detailed molecular architecture of membranes. The proliferation of models of varying molecular detail reflects the lack of agreement that exists concerning the validity of both the experimental evidence and its interpretation. We will here examine the main experimental results and the proposed models which have emerged. The history and literature of the field have been the subject of many recent reviews (15, 39, 62, 83, 99, 132, 164, 225, 229, 245, 252) and no comprehensive treatment will be attempted.

The idea that all cellular membranes might have a common basic structure was first proposed for the plasma membrane. It was based on the demonstrated or assumed functional and compositional similarities in different cells, and was later extended to include the intracellular membranes mainly because all membranes showed a nearly identical appearance in electron micrographs (cf. 222). Furthermore, the simplest cells are known to possess only a plasma membrane and phylogenetically intracytoplasmic membranes are generally thought to have developed from infoldings of the plasma membrane. These are still valid arguments for a common basic structure. Divergent evolutionary trends and the effects of increased functional specialization may, of course, have modified the different membranes considerably and greatly reduced the properties which they hold in common; however, considering the fact that membranes still act mainly as stable barriers between aqueous compartments, one might expect to find that common structural principles can still be recognized.

At present we cannot specify the molecular structure of any membrane in detail. None of the many experimental results and arguments advanced in support of a given structure is compelling. Those which presently occupy most of the attention are listed here in the form in which they are usually discussed in the literature. They can be found in many review articles and special papers, but in our opinion are seldom critically and impartially enough presented. Few authors seem to be aware of all of them. The models which have emerged on the basis of these observations usually seem to be taken too literally. A biological membrane does not have the structure of a model, rather the structural principles exemplified in the model may be recognized in the membrane and its function may be understood, to some extent, on this basis. A critical review may eliminate some misunderstandings and may help to clarify the structural principles which can be presently recognized as contributing to membrane structure and function.

II. PROBLEMS IN THE CHARACTERIZATION OF BIOLOGICAL MEMBRANES

Essentially, we can use three different approaches to recognize and to define a membrane: (a) morphology (electron microscopy); (b) permeability studies; (c) isolation as a separate entity which has retained some or most of the properties ascribed to it from the morphological observations or permeability studies on intact cells or cell organelles. Because electron microscopy does not necessarily demonstrate all components of a membrane and because permeability studies do not give information on membrane components not linked to this function, we do not have any absolute criteria for assessing the completeness of an isolated membrane preparation. It is obvious that, in the isolation process, components only loosely linked to a membrane but essential for its function may have been lost through mechanical forces or changes in the environment and that more firmly bound components may have been lost through enzymic degradation. Such a loss will be noticed only if the other components necessary for this function can be detected in the membrane and only if the function can be restored when the lost component is added to and re-bound by the isolated membrane. The best available solution to this difficulty is to isolate membranes by the mildest available procedures, such as mechanical or osmotic fragmentation and repeated washings with dilute salt or sucrose solutions, and to compare the results of

different techniques. These questions have been investigated and discussed for mammalian cells in general by Wallach (274), for isolated bacterial membranes by Salton (232–234), for the rat liver cell membrane by Benedetti and Emmelot (16), and with special emphasis on the erythrocyte membrane by Maddy (164).

The problem is not too serious for our purpose, because we intend to discuss only the main structural components of membranes which, by definition, would have to be anchored rather firmly in the framework of the membrane, provided the environment does not differ radically from that of the intact cell. It is more important here to resolve the reverse of this problem, i.e., the possibility that during isolation other cell constituents may be adsorbed to the membrane and be mistaken for genuine membrane constituents. This is compounded by the fact that most membranes re-form closed structures upon disruption (274) and may trap soluble or particulate materials inside, which cannot be removed by washing. Again one can only compare variations of the isolation techniques to see which constituents are found consistently in the membrane fraction and which components can be removed without obvious loss of function and changes in morphology. In some cases it is possible to isolate and to label the component in question, add it during the isolation procedure, and see to what extent it is bound to or trapped by the membrane preparation.

Structures which may be called "extraneous coats" are present on the outer surface of cell membranes and are difficult to distinguish from the membrane proper. They constitute a major source of nonmembranous material complicating the analyses of isolated membranes. They are highly developed in the cell walls of bacteria and higher plants where they form a structural and functional complex with the cell membrane, but they do exist in most if not all other cells as well (219). In many instances it is not only physically but also conceptually difficult to separate this complex entity into its components. The reasons for distinguishing between the membrane proper and the extraneous coats are fourfold. First, it can be shown in many cases that the main permeability barrier is located in the innermost layer of the complex. Second, this layer also shows other typical membrane properties such as firmly bound multienzyme complexes. Third, its composition of lipids and proteins and its structure as seen in the electron microscope are very similar in all cells and are also observed in the intracellular membranes. Fourth, most of the material outside this layer can be removed without seriously impairing the viability of a cell, whereas an intact cell membrane is an absolutely essential component of living cells. Since no general compositional or structural principles have so far been postulated for the extraneous coats, except perhaps that they usually seem to contain substantial amounts of polysaccharides, they will not be considered further in this discussion.

Aside from the technical difficulties involved in separating membranes of similar morphology and composition, the recognition of a membrane as to its origin is a very serious problem once the structural organization of the cell has been disrupted. Enzymatic activities are often used as markers for a membrane fraction; other easily recognizable markers may be found on some membranes or bound to them before isolation (274, 163). Possible changes in membrane composition with age or function complicate the problem and it can not, of course, be taken for granted that morphologically apparently uniform and continuous membranes are also functionally uniform over their total extent. Differences in the composition and consequently in the structure of the Schwann cell membrane and the myelin sheath, for instance, must exist even though morphologically they form one continuous membrane. The plasma membrane of many cells shows recognizable structural and functional differences in different parts of the cell surface. These are easy to recognize in epithelial cells which serve excretory and resorptive functions and at points of cell contact in multicellular organisms in general. The same holds true for intracellular membranes, for instance, the grana and intergrana membranes of chloroplasts. Even in bacteria, indications can be found that the plasma membrane is not uniform over the whole surface of the cell at the level of resolution of the light microscope. Data and arguments which are discussed on the following pages may often refer to such areas rather than to the whole membrane that encloses one compartment.

The problems of isolation and diversity limit significantly the confidence with which statements derived from experimentation with isolated membranes can be regarded. For purposes of the present discussion and with the reservations outlined above, it is postulated that the membranes as they are isolated contain the primary structural ele-

ments, even though certain components or properties may have been lost or altered in the process of isolation, and some nonmembranous material may be bound to them.

We shall discuss only the arrangement of lipid and protein in membranes. While an aqueous environment appears to be essential for the stability of biological membranes, only few and conflicting data exist concerning the role and state of water in membrane structure. Furthermore, possible changes of structure in different functional states of membranes will not be discussed. While such changes must occur, it can be shown that in many instances only a small part of the total area need be involved and our techniques are not sensitive enough to detect the change. In the few cases in which we have indications that most or all of the surface may be involved, the data can not be interpreted at present in terms of detailed molecular rearrangement with any degree of confidence (22, 23, 45).

The following brief summary of membrane characteristics includes general properties as well as attributes which are present or have been demonstrated only in limited classes of membranes but which are found only in association with membranes.

A. *Structural Characteristics*

1. Membranes are flexible structures which form closed topological boundaries separating compartments of different composition. They are only a few molecules thick, but they are not merely phase boundaries and can be isolated as separate entities (29).

2. The molecular species present in isolated membranes are predominantly lipid and protein. Much of the lipid is phospholipid, although other amphiphile lipids may be present. In some cells (e.g., plant cells) a large part of the lipid may be glycolipid (17), in mammalian cells the percentage is usually much smaller. A neutral lipid often occurring in considerable quantity is cholesterol; carotenoids and quinones may also be present (90, 230). Small amounts of sugar bound to protein are usually found (on the order of 1–5% of the dry weight; cf. Cook 1968 [47]). The question of whether nucleic acids, especially RNA's, are an essential component of membranes is not resolved at present.

3. Certain physical parameters have similar values for many membranes: thickness, surface tension, buoyant density, electrical resistance, electrical capacitance, water permeability.

4. Cross-sections of membranes consistently show two dense outer bands separated by a lighter central region in the standard preparations for electron microscopy.

B. *Functional Characteristics*

Membranes have been considered for so long nearly exclusively as permeability barriers that it may be useful here to emphasize another aspect by listing it separately, even though these functions are intimately linked to each other. Membranes can function:

1. AS BARRIERS FOR SOLUTES AND WATER: Membranes are in general more permeable to substances soluble in apolar solvents than to similar water-soluble compounds. In spite of a high permeability to water, the permeability to ions is generally low. Large differences in the permeability to different ions, facilitated diffusion, and active (or energy-coupled) transport of ions and small molecules are found in many membranes. Bulk transport of material through pinocytosis and phagocytosis is mainly a property of the plasma membrane but related processes of membrane fusion and separation can be recognized in intracellular membranes. All these processes serve to maintain or to change in a controlled way the compositional differences between the cell interior and its external environment or between intracellular compartments. They appear to be the basic processes for such typical membrane functions as excitability and impulse conduction, secretion, and cell-cell interaction which, however, may also involve other less well understood membrane properties.

2. AS THE STRUCTURAL BASES FOR ENZYMES: Some enzymatic activities are usually found to be firmly bound to isolated membranes, and many of them are difficult or impossible to separate from the membranes without considerable alteration or complete loss of activity. They provide the basis for such typical membrane functions as electron transport and oxidative phosphorylation or photosynthesis. Some enzymes when added to membranes deficient in these components are re-bound, restoring the original enzymatic content and activity.

It should be emphasized again that this constitutes an artificial division of membrane functions into two classes. Enzymes certainly have an

essential role in active transport processes and, at least according to Mitchell's chemiosmotic hypothesis (176), the barrier function is an integral component of oxidative phosphorylation and photosynthesis. The same may be true for another postulated function of the membrane, namely providing a hydrophobic environment for certain processes that could not occur in water (92, 105, 153, 217). The functional aspect will not be discussed in detail. We only intend to establish here that membranes have a set of common physical and functional characteristics which suggest a common basic structure. Moreover, some of the arguments for a general concept of membrane structure are based on the properties of membranes as carriers of enzymes.

III. THE MODELS

Membranes consist mainly of lipid and protein. Their structure should therefore be determined by the lipid-lipid, protein-protein, and lipid-protein interactions which can occur in the aqueous medium that constitutes the internal and external milieu of cells. Of these, the lipid-lipid interactions are best understood and appear to dominate the picture. They form the basis for one model of membrane structure. Little is known about protein-protein interactions of membrane components and their contribution is seldom considered. The lipid-protein interactions are the most hotly debated aspect of membrane structure, in spite of or because of the lack of sound experimental data on the structure of lipoprotein complexes.

The main models that have been presented as applicable to all membranes are the Danielli, or bilayer model, and the subunit model. The latter has emerged only in recent years and has been extensively treated as a general model for membranes by Green and his collaborators (104); similar models have been described more or less independently for a number of specific membranes other than the mitochondrion on which most of the work from Green's groups has been based. We feel that this justifies a general evaluation of the subunit model as an alternative to the Danielli model. Several other models, such as those described by Kavanau (132), Lucy (158), Lenard and Singer (149), Benson (18), and Vandenheuvel (270–273), may be considered variations of one or the other of the two main models and will not be treated in detail; rather, part of the supporting data for these models will be included in the evaluation of the arguments for the two main models.

A. *The Danielli or Bilayer Model*

One of the earliest and most enduring molecular models of modern biology has been the lipid bilayer model for the structure of cell membranes. Originally proposed by Gorter and Grendel (102) and later independently and in more elaborate form by Danielli (53, 57, 58), the central assertion of the model is that membrane lipids are arranged in a sheet roughly two molecules thick in which the polar ends of the lipid molecules are directed outward and the apolar ends inward. Danielli originally proposed, on the basis of surface tension measurements, that protein must be adsorbed at the lipid-water interfaces. Subsequent experiments showed that the oils used in model systems for the lipid-water interface did not accurately represent the properties of a phospholipid-water interface and that phospholipids alone can produce the required low interfacial tension (44, 113, 114); this finding considerably weakened the argument for the participation of protein in the membrane structure. Despite the tenuous evidence upon which the model was originally proposed, it was widely accepted, further developed (29, 54, 56, 59), and has dominated ideas concerning membrane structure for the last thirty years. As a result, a considerable body of relevant evidence has been accumulated. The main points will be presented below, but we shall state in some detail what the characteristics of the lipid bilayer model are as it may currently be understood because certain objections to it derive from a rather rigid view of the model that fails to adequately assimilate recent information.

The central feature of the Danielli model is the continuous bimolecular lipid layer. It is often represented as containing a closely packed bilayer of phospholipid molecules with rigid and fully extended hydrocarbon chains. This would result in a layer with a thickness equal to twice the length of an extended phospholipid molecule. Following the earlier work of Schmitt et al. (239), studies mainly by Luzzati and his collaborators (160, 161) have emphasized the notion that the fatty acid chains of the membrane interior are most likely in a disordered state approaching that of a liquid hydrocarbon. Consistent with this interpretation, the thickness of "bimolecular" layers of membrane lipid formed in an aqueous environ-

ment under conditions similar to those present in living cells was found to be substantially less than twice the length of a fully extended lipid molecule. This also implies that the surface area per lipid molecule in such bilayers must be greater than in a fully compressed monomolecular film which has been widely used as a model system. Calculations from X-ray diffraction measurements show an area of 65 A^2 in the lamellar liquid-crystalline phase of mitochondrial lipids at physiological temperatures as compared with 41 A^2 in a closely packed array (107). Slightly higher areas per molecule have been found in other phospholipid-water systems (213, 246). Studies employing birefringence techniques which measure the order and orientation of the chains in the hydrocarbon layer under similar conditions confirm the disorder for liquid-crystalline phases of other lipids (226). Additional evidence for the liquidlike hydrocarbon chains comes from recent infrared (IR), nuclear magnetic resonance (NMR) and calorimetric studies of membranes and model systems (249). High resolution NMR spectra that can be obtained from the protons in the fatty acid chains of membrane lipids in some membranes and also differential scanning calorimetry show that the melting of the fatty acid chains occurs at the same temperature both in natural membranes and in liquid-crystalline lamellar phases of the isolated lipids. This temperature is generally below the environmental temperature of the membrane in vivo. Cholesterol abolishes these phenomena. The reason for this is still under debate (212, 249). Even if the movement of the chains is restricted in cholesterol containing bilayers, the X-ray diffraction evidence indicates that they are still disordered (86, 213). The disorder and the fluidity in the hydrocarbon region of natural membranes and of model membranes depend mainly on the temperature, the length of the hydrocarbon chains, their degree of unsaturation, and their heterogeneity. That this fluidity is an essential feature of natural membranes is indicated by the fact that in living organisms long-term changes in temperature of the environment appear to be compensated for by changes in the fatty acid composition of the membrane lipids so that the fluidity of the bilayer is maintained (43, 62).

The earlier concept of a bimolecular leaflet with nearly straight and parallel hydrocarbon chains consequently must be modified. The lipid layer should be described as a lipid leaflet with a thickness less than twice the length of a phospholipid molecule and with a disordered central hydrocarbon core bounded on either side by hydrophilic groups which are not closely packed. If the degree of disorder in the central layer is great and/or extensive interdigitation occurs between the fatty acid chains bound to opposite sides of the layer, the total thickness of the bilayer may approach the length of one fully extended phospholipid molecule, and X-ray analysis indicates that such a layer may exist in chloroplast membranes (172).

Since Danielli's original argument for the role of protein layers in the model no longer appears to be valid, the main evidence for their existence is derived from the fact that isolated membranes always contain substantial amounts of protein. It now seems neither necessary to assume that the protein covers the lipid surface completely nor that it is present on both surfaces in approximately equal amounts. The protein has been considered to be in the form of extended monomolecular layers, layers of globular molecules, or a combination of the two (165). The evidence concerning protein structure in natural membranes is most limited at present; however, it does permit the elimination of extended monolayers as the sole or predominant configuration. Such a configuration would be inconsistent with the membrane's function as a carrier for enzymes, with the isolation of native membrane proteins which are irreversibly denatured when fully unfolded, and with recent spectroscopic evidence that at least part of the protein in the membrane is in an α-helical configuration (42, 149, 164–166, 249, 268, 269, 275). The existence of an extended basal protein layer with additional globular proteins bound to its surface has some support from work with model systems which has shown that proteins adsorbed to lipid interfaces may become denatured through unfolding (74, 92, 93, 97); however, there are no definite observations on natural membranes to confirm this idea and the denaturation of protein observed in the model system of Eley and Fraser probably does not occur on membrane lipid bilayers (114). That some conformational changes in proteins will occur when they are bound to membranes is to be expected and can be inferred from the observed differences of activity and specificity of enzymes in the free and in the bound state.

At present there is no reason to assume the existence of a general structural principle for the

conformation of proteins of most or all biological membranes. The binding between protein and lipid usually is depicted as predominantly ionic. The role of hydrogen bonding which is likely to occur has never been established. However, it should be noted that an expanded lipid bilayer as we assume exists in membranes would allow hydrophobic interaction between lipid and protein to occur without disruption of the lipid bilayer (see p. 625, 626).

The binding of protein will have an effect on the structure of the lipid layer as indicated, for instance, by recent NMR data on erythrocyte membranes (43) and an increase in area has been observed when protein is adsorbed to a monolayer of lipid at an air-water interface at film pressures above the collapse pressure for protein monolayers (46, 69, 170). A similar interaction between protein and lipid in a bilayer would be expected to lead to an increase in the area per molecule for the lipid and lead to a decrease of the thickness of the lipid layer. Evidence for this effect in the binding of cytochrome *c* to lipids in the lamellar configuration has been reported (259). For the further discussion it is essential to remember that the area per molecule in biological bimolecular lipid leaflets is substantially larger than that in a closely packed film and that interaction with protein may produce a further increase in area for a membrane with the structure of the Danielli model.

Schematic cross-sections through the Danielli model usually depict it as a symmetric structure. This is clearly an oversimplification. Such important membrane functions as active transport would not be possible in a symmetric structure. Functional differences between the inside and the outside of membranes can readily be demonstrated (1, 122, 243). Early direct evidence for structural differences between the two halves of a membrane came from X-ray studies of the myelin sheath (82, 222, 238). Some enzymes and antigens have also been demonstrated to be present only on one side of the membrane (16, 111) and glycoproteins seem to occur preferentially, if not exclusively, on the outer surface of the plasma membrane (cf. 47). Electron microscopy of stained sections has often demonstrated differences in the density and width of the two outer layers of the unit membrane (73, 245) and the differences may change with the functional state of the membrane (64, 65). Since negative staining and freeze-etching techniques have revealed in more detail the structure of membrane faces, differences in the structure of the inner and the outer surface have been found in most membranes which have been investigated. While these demonstrated differences usually seem to involve only the protein layers of the membrane, it is more than likely that they also extend to the distribution of lipids in the central bilayer. The symmetric representation of membranes in the Danielli model, therefore, reflects only our lack of knowledge about general differences between the two halves of the membrane, and it should not be taken to mean that the model postulates a symmetric membrane.

1. EVIDENCE AND ARGUMENTS SUPPORTING THE DANIELLI MODEL: In this section evidence for the Danielli model from observations of natural membranes will be discussed together with evidence derived from model membranes. We shall list the observation or argument first and then discuss it in more detail. We do not think that all the arguments are necessarily valid or all the observations relevant. However, because they are used as the basis for most present discussions of the model, we feel that it is necessary to summarize them here and try to evaluate their relevance to the problem.

> Chemical analyses of isolated membranes show lipids and proteins to be the main components. They are generally present in amounts compatible with the Danielli model.

While approximately 30–40% lipid and 60–70% protein is a typical value for many clean membrane preparations, the proportion of lipids and proteins reported for isolated membranes varies rather widely and this has been used as an argument against the Danielli model. However, the uncertainties as to the purity of membrane fractions (see p. 614), the possibility of trapped protein or loss of protein make this a rather meaningless argument. Moreover, the amount of protein bound on the surface of the lipid layer is not specified in the Danielli model and may vary considerably in natural membranes. A slightly more meaningful question that could be asked at present concerning this quantitative aspect of the Danielli model is whether enough lipid is present to form a bilayer equal in area to the total membrane area of such a preparation. Aside from the difficulties encountered in measuring this area, to answer the question one would have to assume a value for the surface area occupied by one lipid molecule. This so far has not been established for any natural

membrane. Only for the human erythrocyte membrane has an estimate for the area per lipid molecule been attempted which is based on data obtained with acceptable techniques (11). Here the existence of a continuous bilayer would require an area of $\sim$ 85 A^2 per phospholipid molecule. When compared to values from liquid-crystalline lamellar phases of membrane lipids (160, 213, 246) and other model systems (109) which give values of close to 75 A^2 per molecule, 85 A^2 per lipid molecule appears as a reasonable value provided it is taken into account that in the erythrocyte membrane the interaction with protein may further increase the area per molecule slightly (see p. 619). In the case of isolated mycoplasma membranes, most of the protein of the membrane can be removed through proteolytic enzymes and the remaining protein-depleted vesicles still show osmotic properties (249). These observations argue for the possibility that a continuous lipid bilayer could exist over most of the surface. This is the type of argument which we think is not really pertinent in a discussion of the general principles determining membrane structure. The bilayer does not have to be present over the whole surface of any membrane to establish the relevance of the model for an understanding of biological membranes.

> Membranes generally are more permeable to small molecules with a high oil-water partition coefficient than to those with a low coefficient.

This was first established by Overton (196). It indicates that a part of the permeability barrier may be a hydrophobic region presumably formed by the lipid. It does not exclude that another substantial part of the barrier may be mainly formed by proteins. However, there are some indications that the action of phospholipases and organic solvents in low concentrations generally results in a nonspecific breakdown of the permeability barrier, whereas the action of proteinases rather will tend to result in a breakdown of specific functions and not so much affect the general properties of the membranes (264) (see also p. 626). This would be consistent with the assumption that the primary permeability barrier is formed by the lipid. These observations may deserve some closer scrutiny.

> Results from studies of myelin structure using birefringence measurements, X-ray diffraction, and electron microscopy are all consistent with a structure composed of bilayers of lipid and protein which are formed by wrapping many turns of membrane concentrically around the axon. This membrane is continuous with the plasma membrane of the Schwann cell. The well-supported structural model of the myelin sheath, when extrapolated to the Schwann cell membrane, results in a molecular arrangement identical to that proposed in the Danielli model.

Following the early work of Nageotte (188), W. J. Schmidt (236), and F. O. Schmitt (237, 238), the structure of the myelin sheath can be considered to be better established than that of any other membrane or membrane-related structure (83, 84, 177, 222, 282). This has therefore been considered as one of the strongest arguments for the Danielli model. It has, however, been repeatedly stated that the composition of the myelin sheath is not typical for cell membranes both with regard to the lipids found and the lack of demonstrable enzyme activity (4, 137, 193). While this may be true (the lipid composition of very few cell membranes is known and that of the Schwann cells is not among them), a difference in lipid composition does not necessarily imply a basic difference in structure.

The main function of the myelin sheath seems to be that of an insulator for portions of the nerve axon. The basic functional characteristic of a Danielli-type membrane is a low electrical conductivity. This could be achieved with bilayers of quite different lipids. The impedance measurements on cells show the same basic properties for the cell membrane (see p. 621), and the electron microscopic observations indicate no major structural change in the transition from Schwann cell membrane to myelin sheath. Moreover, the liver cell membrane, which is the one cell membrane other than the erythrocyte membrane that has been isolated in pure form and rather carefully studied, has a lipid composition qualitatively similar to that of the myelin sheath (16). Therefore, this is still considered to be a valid argument for the Danielli model.

> The three-layered "unit membrane," structure demonstrable in electron micrographs of thin sections for most membranes can readily be interpreted as representing a Danielli-type structure.

This argument involves a comparison of model membranes and natural membranes. Model mem-

branes can be formed in water from isolated membrane lipids and proteins. When ordered structures of such systems are obtained, X-ray diffraction evidence indicates that their structure corresponds to the Danielli model. In the electron microscope they appear as unit membranes indistinguishable from natural membranes (254, 256, 257, 259). The main objection to this argument centers around the explanation of the observed density distribution, in terms of the chemical reactions taking place during fixation and staining. These can not at present be detailed. It must be remembered that fixation and staining are not necessarily identical processes in electron microscopy as is sometimes assumed. While the main action of OsO_4 as a fixative probably occurs through cross-linking of lipid molecules at the site of the double bonds, the main accumulation of heavy metals may well occur around the hydrophilic groups of the lipid molecules and protein layers as required for the explanation of the unit membrane structure in terms of the Danielli model (for a more detailed discussion see p. 624).

Closely related to this problem is the question whether the discontinuous appearance of the dense lines in electron micrographs of the unit membrane corresponds to a real structure in the membrane. This cannot be used as an argument against the Danielli model as will be further discussed in connection with the subunit model (see p. 625).

The variations in the dimensions of different membranes that are observed in cells can be explained on the basis of differences in lipid composition and the amount and stainability of the protein in the surface layers. For instance, endoplasmic reticulum or mitochondria, which consistently show a narrower unit membrane than the cell membrane, contain lipids with shorter and more highly unsaturated fatty acids than the plasma membrane or the myelin sheath (for a review see Fleischer and Rouser [90]). The thickness of the dense layers of the unit membrane varies considerably according to the heavy metal used in the fixation and staining procedures. Similar differences are obtained in model systems when different lipid extracts and proteins are used (259).

> Impedance measurements on suspensions of cells and isolated cell organelles are compatible with the assumption that they are bounded by a continuous hydrocarbon layer of the same thickness found in phospholipid bilayers.

This fact was first established for erythrocytes by Fricke (96) in extension of earlier experiments by Höber (118–121). Similar results were obtained more recently with other cells (241) and cell organelles, for instance, isolated mitochondria (210, 211). The assumption made is that the hydrocarbon phase in a lipid bilayer has the same dielectric constant as the bulk phase of a fluid hydrocarbon. This might not hold true if the hydrocarbon chains of the lipid molecules were highly oriented as they are in the classical bimolecular lipid layer. However, X-ray diffraction evidence indicates that, in the myelin sheath and in isolated membrane preparations (86), the hydrocarbon chains are disordered. NMR (41, 212, 249), IR (38, 42), spin-labeling (125), and differential thermal analysis studies (43, 249, 251) also point to a disordered state for the hydrocarbon parts of the lipid molecules, at least in some of the few membranes so far studied. While the impedance measurements indicate that the layer with the low dielectric constant covers most of the surface, it should be emphasized that small discontinuities in this layer might not be detected by this technique. Nevertheless, this appears as one of the strong arguments for the Danielli model.

> Freeze-etching confirms the gross structure and dimensions of membranes obtained with other techniques and reveals a preferential splitting of membranes in a central plane.

This new preparation technique for electron microscopy is based on rapid freezing of specimens at liquid nitrogen temperature and the observation of replicas obtained from freshly exposed surfaces in the frozen state (179). It overcomes many of the objections that can be raised against electron micrographs of fixed, dehydrated and sectioned or negatively stained specimens. The splitting of membranes along a central plane, which has been observed under these conditions, is rather easily understood in terms of the Danielli model, which postulates a concentration of the terminal CH_3 groups in this plane. No such plane is obvious in the subunit models proposed so far. While the question, which face of a membrane is exposed by freeze-etching, is still somewhat controversial and may vary with the experimental conditions, the specific cases discussed by Branton (24) appear to be well supported. Moreover, cleaving of authentic lipid bilayers in the frozen state results in separation into two monolayers, demonstrating a cleavage plane identical to that

found in freeze-etched natural membranes when a Danielli-type structure is assumed for the latter. These lipid bilayers are formed between two supporting slides from monolayers at an air-water interface (61). No natural lipids have thus far been used in these experiments. The hydrocarbon chains of the bilayers derived from surface films should be in a more regular arrangement than those found in the mixed lipid bilayers from natural membranes and this may be the reason for the less regular cleavage observed by Staehelin (248) with multilayered systems of natural lipids. That in other cases a splitting of natural membranes may occur along the outer or inner membrane does not, of course, argue against the Danielli model.

> Lipids isolated from membranes and dispersed in water tend to form bilayers as the most stable configuration. The physical parameters of such bilayers are very similar to those of natural membranes.

If the lipid bilayer is regarded as the primary structural element in membranes, it must be thermodynamically stable. The lipid bilayers in the lamellar liquid-crystalline phases of natural lipids have been found to be stable over a wide range of temperature and lipid-water ratios (160, 247). This can also be deduced from theoretical considerations (56, 56a, 66, 208). The physical properties of the bilayers appear to be relatively little affected by the thickness of the water layers which separate them. Moreover, they are not only stable in a liquid-crystalline phase but at higher water concentration vesicles are formed that are bounded by a layer of lipid with the same dimensions and presumably molecular arrangement as the bilayer in the lamellar liquid-crystalline phase (259). The bilayers occur at temperatures and water concentrations close to those found in living systems and appear to be much more likely to occur in natural membranes than the other structural elements found in liquid-crystalline lipid-water systems, namely the cylindrical rods. Since the description of the different liquid-crystalline phases, considerable attention has been given to speculations that structure changes from lamellar to rod-like, similar to those occurring in lipid-water systems, may also occur in membranes and might account for some of the functional permeability changes observed in membranes. It should be pointed out that stability for the configurations containing rodlike elements has only been demonstrated in bulk phases. The conditions encountered in a natural membrane less than 100 A thick are so different that conclusions from bulk phases with regard to their structure should be drawn with some caution. For the bimolecular leaflet the lamellar liquid-crystalline phases offer a system by which it can be studied conveniently, but its implications for membrane structure are based mainly on its demonstrated stability outside the bulk phase.

Other lipid-water systems have been described by Lucy and Glauert, Bangham and Horne, Dourmashkin (cf. 99), but they are only observed in electron micrographs of negatively stained specimens. No information on their configuration in solution is available. Even though it may seem attractive to speculate about possible roles of such structures in membranes, some direct evidence for their existence in membranes is required before they can be discussed seriously in this context.

The liquid-crystalline lamellar phase or more highly dispersed bilayer systems have been studied extensively as model systems for membranes. The electron microscopy, X-ray diffraction, and permeability data are discussed on p 620, 617, 618, and p. 623 respectively. In the last few years spin labeling, NMR and calorimetric studies have been added (cf. 249). In general the results can be interpreted as support for the Danielli model (38, 40, 249). However, a word of caution should be added here. Since the bilayer appears to be the favored structure for the lipids, a breakdown of the biological membranes or model systems during the experiment would usually lead to a formation of bilayers in the form of myelin figures. This is a well-known phenomenon to morphologists. At the end of the calorimetric, spectroscopic, or X-ray diffraction experiments, it has therefore to be established that the membranes or model systems investigated are still intact and that no rearrangement to the thermodynamically favored lamellar phase has occurred. Unfortunately, this is usually neglected. Similarly, as has already been pointed out (255), during processing for electron microscopy it is necessary to establish that the fixation, dehydration, and embedding does not introduce the formation of the bilayer configuration.

> Lipid bilayers in an aqueous environment will interact with proteins while retaining their lamellar structure and will assume a three-layered appearance indistinguishable

from natural membranes in electron micrographs of fixed and stained thin sections. Artificial membrane-like lipoprotein structures have been demonstrated mainly by electron microscopy (256, 259). X-ray diffraction evidence is also available to show that the techniques used in the preparation for electron microscopy do preserve the lamellar structure at least within the limits of resolution of the electron micrographs (259, 263). Furthermore, highly dispersed lipoprotein preparations form closed structures of the same size and general appearance as those usually obtained when cell membranes are fragmented, i.e. vesicles of a few hundred to a thousand Angstrom diameter, bounded by a triple-layered membrane.

Thin films of membrane lipids which approach the thickness of natural membranes and the structure postulated for the bilayer model have been shown to approximate the permeability properties of cell membranes. Monolayers of lipid at an air-water or oil-water interface have, for a long time, been the main source of information for the properties of lipid films of molecular thickness. Since this work recently has been reviewed extensively (6, 159) and since the application of the data to an understanding of natural membranes appears to be limited (55, 66, 114), we shall not consider them here. This discussion will be restricted to model systems assumed to consist of bilayers separating two aqueous phases.

Very thin lipid films formed between two aqueous compartments which are both accessible during the experiment were first explored by Mueller, Rudin, and their collaborators (184, 185). They offered the opportunity to study many functional properties of thin lipid films and relate them to cell membranes (e.g., 13; for reviews see 265, 266). It was found that the water permeability and electrical capacitance were close to that of cell membranes but that the permeability to ions and consequently the electrical conductivity were orders of magnitude lower. The films, when formed from membrane lipids, did not show the discrimination between different ions observed in the membranes from which they were derived (2). Their thickness appears to be somewhat higher than that of a bilayer of phospholipid, probably caused by the presence of a considerable quantity of the hydrocarbon usually used as a solvent for the lipid (3, 6, 115, 116). There is little doubt, however, that the orientation of the phospholipid molecules in these films is the same as in a bilayer. Their electrical properties seem to be largely determined by the presence of the central hydrocarbon layer (110). Additives such as protein and peptides lower the electrical resistance of these films to values comparable with those of natural membranes (155, 185, 267). They appear to set up sites in the film which have a high permeability for ions. These sites may be either fixed pores or carrier mechanisms and they may display a rather high specificity for and discrimination between small ions (72, 154, 242). The specificity of the sites is also indicated by the observation that the additives may increase the electrical conductivity by several orders of magnitude without a concomitant increase in water permeability (36). That the sites occupy only a very small percentage of the total area of the film can be deduced from the fact that the capacitance of the system does not change significantly when the ion permeability increases several orders of magnitude (114, 183). It has been possible, by introducing a combination of specific sites, to mimic some of the typical properties of cell membranes in such modified thin lipid films and to elicit, for instance, electrical responses analogous to action potentials in natural membranes (183) or to build a model for a temperature-sensitive receptor (87).

Another model system of lipid bilayers has been developed by Bangham and his collaborators (6–10, 159, 200, 201). It consists of a dispersion of phospholipid in water and has the advantage that only phospholipids or other amphiphile lipids are necessary for its formation. Other advantages of this model system are that osmotic properties can be followed by light scattering and that the large area of the lipid film as compared to the Mueller-Rudin type black films makes measurements on the permeability to uncharged and slowly permeating molecules easier in this system. Its main disadvantage is the somewhat uncertain geometry that generally allows only qualitative conclusions to be drawn about the action of additives and even casts some doubt on values for the relative permeability to different solutes. Nevertheless, it has been shown that these lipid bilayers react to added drugs and other modifiers of permeability qualitatively in the same way as natural membranes. Moreover, the data obtained, while differing in some respects, are generally similar to those from Mueller-Rudin

model membranes (198) and confirm that the additional hydrocarbon probably present in the latter model does not affect the relevant properties to a very great extent. Both model systems argue strongly for the possibility that a lipid bilayer could form an essential component of biological membranes, especially since many of the agents which modify the permeability of the model systems have a similar effect on natural membranes. (For a general review of these model systems see refs. 6, 265.)

These are the main experimental data and arguments that support the Danielli model. Arguments and data that have been held to be incompatible with this model or at least suggest alternate explanations for some of the observations will be enumerated and discussed in the following section. Evidence advanced in favor of a subunit structure of membranes, e.g. the disaggregation of membranes into apparently identical lipoprotein particles which has been considered as evidence against the Danielli model, will be presented in connection with the subunit model.

2. EVIDENCE AND ARGUMENTS AGAINST THE DANIELLI MODEL: Many arguments against the Danielli model are based on the assumption that the model is meant to describe accurately and completely the structure of biological membranes. This is not, of course, the purpose of the model. It would be equivalent to demanding that all proteins must be α-helical and all nucleic acids double-helical for these structures to be acceptable as relevant models of protein and nucleic acid conformation. In our view, the bilayer model only postulates that the assembly of membrane lipids into a bilayer is a dominating factor governing membrane structure and that proteins associate on and with the lipid layer to form a complex in which the gross morphology is largely determined by the lipid. While this interpretation of the model renders many of the often-heard arguments against it invalid, some of these are still listed here because the observations on which they are based are of interest and possible interpretations should be discussed.

> The chemical reactions of OsO_4 with lipids have been held to be incompatible with the appearance of the Danielli model as a triple-layered unit-membrane structure in electron micrographs of OsO_4-fixed cells and tissues.

That the first reaction of OsO_4 with most unsaturated lipids occurs at the site of unsaturation and follows the reaction scheme described by Criegee has been postulated on the basis of indirect evidence (258, 261). The final reaction product, a diester that forms an intermolecular link between two lipid molecules, has been shown to occur when unsaturated lipids react with OsO_4 in water (14) and has been recently isolated in model experiments using membrane phospholipids (138). This cross-linking of molecules probably forms the basis for the reaction of OsO_4 as a fixative for most unsaturated lipids. However, for every diester bond formed, one osmium atom in the form of a lower oxide is produced and its further reactions are uncertain. Moreover, some phospholipids may also react with OsO_4 through their hydrophilic group, e.g., phosphatidyl serine (261). Finally, the triple-layered appearance of cellular membranes is most consistently observed after additional staining with heavy metal salts, which are most likely to react with the hydrophilic groups of the phospholipid molecules. A reaction only at the double bond would certainly not be consistent with the unit membrane appearance. However, the additional reactions of osmium and heavy metal stains could easily explain the observed triple-layered structure but will have to be explored in detail. In any case, the fixation and staining of authentic bilayers of membrane lipids and lipoproteins in cases where the preservation of the lamellar structure has been demonstrated by X-ray diffraction (259, 263) clearly shows that the triple-layered structure seen in micrographs is compatible with the Danielli model. For natural membranes the myelin sheath has so far offered similar evidence (80) and recent studies on isolated membrane fractions both with X-ray diffraction and with electron microscopy (86) also confirm this conclusion.

> Often the triple-layered unit-membrane structure is not demonstrable in electron micrographs of membranes. They may appear as single dense lines or the two dense lines of the unit membrane may be replaced by rows of more or less regular dense granules and/or dense cross-bridges may be present between the lines or rows of granules.

A single dense line may arise for several reasons which have been discussed in detail by Elbers (73) and Sjöstrand (244). This appearance can be interpreted on the basis of differences in the stainability of an asymmetric membrane and does not argue against the Danielli model.

Also, a unit membrane structure in which rows of granules appear instead of two dense lines still can be interpreted on the basis of the Danielli model. The distribution of density along these lines would then reflect the presence of groups that preferentially bind the heavy metal, and this appearance cannot exclude the presence of continuous lipid and protein layers. That the same appearance also may arise as an artifact in the electron microscope has been demonstrated (257). The cross-bridges observed between the two dense lines may similarly be explained as an artifact (15, 16, 224). This reduces considerably the value of these observations as evidence against the Danielli model. Nevertheless, there is no conclusive evidence that these structures are indeed artifacts and they certainly deserve a closer examination. However, their relevance for the present discussion appears to be rather limited until the doubts concerning their reality have been removed.

> The triple-layered appearance of membranes seen in electron micrographs is found in apparently membranous cell structures that, because of their chemical composition, cannot have the structure of the Danielli model.

The most prominent examples for this are the outer layer of the cell wall in gram-negative bacteria (128, 231), the collapsed gas vacuoles of halobacteria (260), a cyst wall component in *Fasciola hepatica* (68, 175), and the lipid extracted membranes of the myelin sheath (189) and mitochondria (89). The fact that different molecular arrangements may give an essentially identical appearance in the electron microscope does not, of course, preclude the interpretation of the unit membrane on the basis of the Danielli model; it only reduces the weight of this argument as support for the bilayer model. This is not too serious in the case of the outer layer in the gram-negative cell wall where recent X-ray diffraction studies indicate that the wall may have a structure analogous to the Danielli model in which only the protein layers have been replaced by polysaccharide layers (31–33).

The cases of the cyst walls and gas vacuole membranes present a more serious challenge because no lipid has been found in these structures. These cases demonstrate, moreover, that stable structures of the general morphological characteristics of cellular membranes can exist that are free of lipid. This appears to be a rather important observation, because so far only lipids or lipoproteins have been known to give rise to membrane-like structures under conditions similar to those of the cellular environment. The question arises about how these structures are formed. At present it seems still possible that they are originally assembled with the help of lipid that is later removed or that the gas-water interface plays a decisive role at least in the halobacterium membranes, but there is no evidence for this and the problem should be further pursued.

The last two examples cited belong to a different category again, because here the lipid has been artificially removed. In the case of the myelin sheath, it may still be postulated that the preceding fixation has stabilized the morphology of the protein in this multilayered concentric structure to such a degree that in the subsequent embedding procedure the embedding medium simply replaces the lipid and keeps the structure expanded. Such an argument is not possible in the case of lipid-depleted mitochondria which have not been fixed prior to lipid extraction, and a fixation effect of the solvent used in the extraction is highly unlikely because electron transport activity is restored upon addition of lipid to the preparation. Here a modification of the Danielli model is clearly required to explain the findings, and cross-bridges between the two protein layers have been suggested as the minimal change compatible with the observation. However, it should be pointed out that this observation does not necessarily favor any other membrane model and rather tends to confirm one essential feature of the Danielli model, i.e., the existence of the protein component mainly in the form of continuous sheets on both surfaces of the membrane.

> A hydrophobic interaction of membrane proteins with lipids can be observed in isolated membrane components and may also occur in natural membranes.

Not all the evidence advanced in support of a hydrophobic interaction between lipids and proteins is compelling. For instance, the argument that membranes cannot be dissociated into lipid and protein in high salt concentrations assumes that the ionic groups responsible for the binding are freely accessible, which may not be the case. Most observations on the dissociation of membranes through the breaking of hydrophobic interactions do not distinguish between the three possible hydrophobic interactions, lipid-lipid,

protein-protein, and protein-lipid. Other evidence is based on the isolation of so-called "structural protein" from membranes. This is an apparently heterogeneous (108, 151) protein fraction that was originally isolated from mitochondrial and chloroplast membranes (27, 48, 49, 278, 279) and later in similar form from a variety of other membranes as well (103, 240, 277, 281). "Structural protein" shows strong hydrophobic interaction with itself and with lipid (221). Its role in the membrane has never been unequivocally established and recently it has even been questioned whether it is really a membrane component in mitochondria (91). Some of the evidence for hydrophobic interaction between lipid and protein derived from optical rotatory dispersion (ORD) and circular dichroism (CD) data on membranes (149, 276) recently has been shown to be invalid because the observed spectral shifts can be produced by protein alone (250) and can be explained as a scattering artifact (268, 284).

At present it appears impossible to quantitate the contribution of ionic and hydrophobic interaction to the stability of membranes (60). Even assuming that extensive hydrophobic interaction between lipid and protein exists in membranes, this argument against the Danielli model appears valid only if the representation of the model as containing a densely packed bimolecular lipid leaflet is considered. However, as pointed out on p. 617, there is good reason to assume that the area per lipid molecule in membranes is considerably larger than in a closely packed lipid film. This would result in a wider spacing of the hydrophilic parts of the lipid molecules. As already pointed out (see p. 619), reaction with the protein may further increase the area per lipid molecule, and hydrophobic interaction of lipid hydrocarbon chains with hydrophobic areas on the surface of folded protein molecules appears possible without disruption of the lipid bilayer. Moreover, some penetration of hydrophobic parts of protein molecules into the central hydrocarbon phase of the lipid bilayer may well occur and is not incompatible with the Danielli model as we see it. Data that have been interpreted as evidence for an interaction between proteins and the fatty acid chains of lipids in bilayers come from recent NMR studies of model membranes (43) and are also compatible with this model. Work on the enzymic conversion of the monounsaturated fatty acid chains of phospholipids into cyclopropane chains are also of interest here. This reaction takes place in bacterial membranes and can be duplicated in vitro with isolated enzymes and lipid bilayers. Apparently, it does not involve a removal of the fatty acids from the bilayers (147). Sharp breaks in the temperature curve for the activity of these enzymes indicate that the physical state (probably the mobility of the fatty acid chains in the bilayer) is of importance. This interesting system deserves further study and correlation with data on membrane structure (cf. 152).

> The ratio of protein to lipid constituents found in isolated membranes varies widely, and the myelin sheath for which the Danielli structure seems to be best supported is found at one extreme in this range of compositions.

This often-raised objection, in our opinion, is not a valid argument as has already been discussed on p. 619.

> Phospholipases extensively degrade the phospholipids of native membranes, which should be protected by the protein layers on the surface of a Danielli membrane, whereas the degradation of proteins by action of proteinases on native membranes is rather limited in many instances.

This argument (e.g. 150) would lead to a membrane model in which the lipid is concentrated on the surface and most of the protein is in the interior of the membrane. We see no reason why the lipid in a bilayer membrane should be inaccessible to lipases. Apparently, it is available for modifying enzymes and for hydrophobic interaction in general (see p. 625). Thermal motion in the membrane may be sufficient to expose the lipid to the soluble proteins in the surrounding medium or more permanent gaps in protein layers of the membrane may exist. It is also possible that some membrane protein may be exchanged for the lipases.

The limited digestion of membrane protein by proteinases may be explained by restrictions in orientation and configurational changes in the membrane proteins. Examples of such an inhibition of enzyme activity are known for nucleoproteins and are consistent with our concepts of the mechanism of enzyme action. In agreement with this interpretation is the observation that unspecific proteases, which can cleave any peptide bond, will digest most of the membrane protein (249). Moreover, membranes usually form closed structures and we may assume that in

many instances the protein on the interior surface or contained in soluble form in the vesicles will not be accessible to the protease added on the outside and one should expect only a limited digestion. If polysaccharides or related compounds are present on the outer surface, these may further restrict the accessibility of the membrane proteins for the enzyme.

> Electron microscopy of freeze-etched cells shows particulate structures in or on membranes, which have been interpreted as globular lipoprotein complexes that extend through the thickness of the membrane.

The images of membranes in freeze-etched preparations are still difficult to interpret but in general tend to support the structure deduced from electron micrographs of fixed embedded and sectioned material (see p. 621). While it appears reasonably certain that in some cases membranes split along a central plane, it also appears likely that under other conditions the exposed surface is actually the inner or outer membrane surface (248). In the latter case, there would be no contradiction in the existence of globular particles on the membrane surface and the Danielli model. In cases where globular particles appear to be embedded in membranes that have split along a central plane, this splitting can be used as an argument for a Danielli-type structure and the globular particles would then have to be interpreted as regions with a different structure. However, possible artifacts that may arise during the freezing and fracturing of the specimen or afterwards through contamination of the surface or changes during the shadowing will have to be investigated before these pictures definitely can be accepted as a reliable image of membrane fine structure.

> Since the Danielli model, especially in its extended form in the unit membrane theory of Robertson, holds that all membranes have the same structure, it cannot possibly explain the functional diversity found in natural membranes.

Its inability to explain most of the biological functions of membranes is the most serious limitation of the Danielli model. It absolutely requires some qualifications to its general applicability. Structural modifications will have to be introduced that in all probability will not be confined to the protein layer but will extend to the lipid bilayer. However, when specific functions are investigated it can often be shown that they seem to be dependent on relatively few sites that occupy a negligible percentage of the total surface area. Thus, there are about 300 virus receptors on a bacterial cell and it has been shown recently that the part of the receptor which involves the membrane may be the same for many different viruses (12). The maximum number of sites of K^+ transport in the human red cell has earlier been estimated as 1,000 (100) and more recent experiments suggest that there may be actually only 100 to 200 sites (75, and J. F. Hoffman. Personal communication). The calculated total area for water channels in the red cell membrane is also small compared to the total surface area (197). The maximum number of Na^+ channels for the axon surface is estimated at $13/\mu^2$ (180). Transport proteins in bacterial cells occur in relatively small numbers ($<10^4$ molecules per cell) even under conditions by which maximal amounts are produced, and only very few proteins seem to be involved in the transport mechanism (202). The electrical properties of cell suspensions and tissues offer a more general argument for the same conclusion (241). In model systems in which additives are used to induce permeability to ions comparable with that of natural membranes, again only a negligible percentage of the total film area appears to be modified (113, 183). Therefore, it seems possible that a substantial part of many membranes still may be dominated by the bilayer structure, while specific functions are carried out by modified sites, which may occupy only a small percentage of the membrane area.

3. GENERAL EVALUATION OF THE DANIELLI MODEL: Today the studies on the association of membrane lipids and proteins in water form the main basis for the Danielli model. These and most of the other arguments for and against it have been presented here. It is obvious that none of them is compelling; however, the number of similar observations from a wide variety of biological membranes that are compatible with the bilayer model is rather impressive. The chemical composition of isolated membranes, their structure as it appears in electron micrographs or can be deduced from X-ray diffraction data, their electrical properties, the similar reactions of model bilayers and natural membranes to modifiers of permeability, and the general functional characteristics are all readily interpretable in

terms of a basic bilayer structure. Most of the counter arguments can be discounted because they are either based on misconceptions of the model or are insufficiently supported by experimental evidence. None of them appears to exclude, for natural membranes, the dominating role of the structural principles exemplified in the bilayer model or clearly suggest alternative structural principles. The possible role of a subunit structure will be discussed in detail in the following section.

The bilayer model has proved useful mainly for the development of model systems. So far the functional consequences resulting from a central liquid-like or disordered continuous hydrocarbon region have not been explored very far. It has been considered mainly as a permeability barrier but other aspects have been neglected, such as its mechanical properties, change with temperature, or the influence of a low dielectric constant environment on the physical and chemical processes that may take place in membranes. While the model has been rather successful in describing the role of lipids, it does not as satisfactorily deal with the proteins. It is clear that the proteins contribute significantly to the stability of the bilayer, but the nature of the protein-lipid interaction is not sufficiently well defined and nothing is said about the protein-protein interaction. Most of the work on model systems has concentrated on lipids, which was a logical first step since the model attributes a dominating role to lipid-lipid interactions. However, model systems containing lipid and protein are now investigated in several laboratories. They can be expected to yield some insights into the principles governing the lipid-protein and protein-protein interactions.

One of the major shortcomings of the bilayer model is that it does not readily suggest any crucial tests of its validity. This feature of the model has allowed it to exist for over thirty years without definitive confirmation or rejection and certainly accounts for much of the present dissatisfaction with it. Only in recent years have serious attempts been made to test natural membranes for the presence of lipid bilayer structure with advanced spectroscopic and calorimetric techniques. So far the results have been generally favorable for the model but again not decisive (see p. 622). It seems possible that work on the model systems will lead to sufficiently stringent criteria for these or related techniques to allow a quantitative determination of the amount of bilayer actually present in natural membranes. This would be a major step in clarifying the extent to which the bilayer model is relevant for biological membranes.

B. The Subunit Model

The successful use of the subunit concept in the elucidation of virus structure and the discovery that large protein molecules often consist of subunits probably have been factors in the attempt to explain membrane structure on the basis of a similar concept. For the organization of entities which are large in comparison with molecular dimensions, a subunit structure has the obvious advantage that it reduces greatly the genetic information necessary to specify the structure. Self-assembly and amplification effects through cooperative changes in structure are easy to understand and are expected to occur. These advantages obtain only if one or a few classes of identical subunits are present and this is what is generally postulated for membranes. The term "subunit" has been well defined as it applies to viruses (35); the same cannot be said for membrane subunits. The following is an attempt to separate the different meanings apparently implied in its use in connection with membranes and to establish some criteria for the definition and identification of subunits.

POSSIBLE CATEGORIES OF SUBUNITS: It seems useful for our purpose to distinguish between subunits of structure, subunits of function, and subunits of assembly. In many instances it may appear to be more logical to use the term "unit" rather than "subunit," especially when considering subunits of function or subunits of assembly. One can, however, argue that the whole membrane enclosing one compartment is the unit, which has a structure and function that cannot be served by single subunits, but only through their assembly into a closed surface. Moreover, the term subunit has been so widely accepted that attempts to change it appear futile, at least for the structural subunits, and if it has to be accepted for the subunits of structure it appears advisable to extend it also to the subunits of function and assembly.

a. Subunits of structure: One would expect that membrane subunits, unlike the subunits of virus capsids or enzymes, are in general supramolecular

complexes of different chemical components because a wide variety of such components is known to be present in most cellular membranes. Membrane subunits are usually thought to consist of lipid and protein. It is also conceivable that the lipid and the protein parts of a membrane consist of separate subunits, or that only one of the two has a subunit structure. However, one has to postulate that the composition of the subunits closely reflects the overall composition of the membrane or its major parts, so that only a few classes of subunits would exist in any membrane. If too many different kinds have to be postulated, the subunits could only form a part of the membrane and another common structural principle would have to be invoked which could link these to form the membrane continuum. At present it is common practice to call any particle of macromolecular dimension a subunit (or an elementary particle) if it has been isolated from a membrane or observed on it in electron micrographs, even when it can be shown to be a single enzyme molecule; examples are the adenosine triphosphatase (ATPase) of mitochondria (259) or the saccharidases of the microvillus membrane in the intestinal epithelium (129, 130, 194). This use of the term subunit is of no help in our attempts to understand membrane structure.

The term "structural subunit" might comprise two classes of subunits that have been distinguished in the case of virus subunits, namely chemical and morphological subunits. The multitude of chemical components and our inability to separate and to reassemble them prevents such a distinction in membranes. It might appear necessary to consider the possibility that membranes could consist of morphologically similar subunits of different chemical composition which possess a set of common binding sites in identical or nearly identical geometric arrangement. However, this appears incompatible with the basic principles of subunit structure; moreover, these are purely hypothetical considerations. The term "structural subunit," as it is presently used, simply implies the existence of one or of a few classes of lipoprotein particles linked through identical or equivalent binding sites which constitute the bulk of the material of a membrane and determine its characteristic shape.

b. Subunits of function: The definition of a functional subunit has to be considered here, because one of the strongest arguments for a subunit structure o membranes can be derived from this concept. While the term "functional unit" was used long before structural implications were considered and may be preferable, the idea has become so intimately involved with the subunit hypothesis that for discussion in the present context it is better to use the term "functional subunit." A functional subunit would comprise all the components necessary to carry out a given complex function of a membrane and different functional subunits may be present in one membrane. Those components that are not bound to the membrane for an extended time and those that only serve to establish a general permeability barrier will not be considered parts of functional subunits although the latter could be structural subunits. The components of a functional subunit need not be contiguous. However, if it can be shown as it has been postulated for the mitochondrial membrane, for example, that these components occur in fixed numerical proportions, exist in a closely packed and bonded spatial arrangement, and constitute a substantial portion of the total membrane material, such a functional subunit would also constitute a structural subunit. Actually, one of the first subunit models for a membrane has been proposed for the mitochondrial membrane (105, 106, 148) on the basis of such considerations.

The definition of a functional subunit is necessarily somewhat arbitrary. The sequence of reactions involved may be broken up into several linked "functions" and the difficulties in determining whether a component is bound to the membrane or not have already been discussed. Moreover, the possibility that components are bound to but can freely move in the membrane has to be considered. Therefore, it seems best, as has already been suggested (259), to reserve the general term "subunit" for cases in which structural and functional subunits coincide. In all other cases a distinction between structural and functional subunits should be made, and arguments for one do not necessarily support the other.

c. Subunits of assembly: The possibility that membranes may be formed in vivo from lipoprotein particles through a self-assembly process has been considered as one of the most attractive features of the subunit model. It has even been stated that it is the only way in which membranes could be formed (137). This is not true, of course,

and the formation of membrane-like structures in aqueous suspensions of finely dispersed lipids, in the form of bilayers which may bind subsequently added soluble proteins at their surfaces, clearly indicates another possible mechanism. Nevertheless, the reported disaggregation of membranes into lipoprotein particles and their reconstitution into membranes has been one of the main arguments for a subunit structure, which necessitates that the possible existence of subunits of assembly be discussed and that their relation to structural subunits be clarified.

While one might expect that in a membrane consisting of structural subunits the same subunits may also be subunits of assembly, this assumption would still have to be verified. It is conceivable that the subunits might be assembled within or on the membrane from their components. Considering the reverse case and assuming that new membrane is formed from subunits of assembly, it does not necessarily follow that the completed membrane has a subunit structure. One would still have to demonstrate that the subunits of assembly persist as recognizable entities in the intact membrane. In other words, the concept of subunits of assembly is not necessarily linked to the concept of subunit structure and independent evidence is required to establish the existence of both for any given membrane.

Very little is known about the biosynthesis of membranes; however, when it has been investigated, for instance in the endoplasmic reticulum of the liver cell (51, 52, 195, 199), the results do not favor an assembly from lipoprotein subunits and the turnover rate of lipids and proteins was found to be different. To our knowledge, no data on the biosynthesis of other membranes indicate that subunits of assembly exist and, so far, little light has been shed on membrane structure from studies of membrane biosynthesis.

In general the ability to form extended thin layers less than 100 A thick is well established for lipids and the structure-determining forces are reasonably well understood. The same cannot be said for proteins, unless a preformed interface exists on which the protein can be arranged. Two possible exceptions to this, the gas vacuoles of *Halobacterium halobium* and probably also of some blue-green algae and the cyst wall components of *Fasciola hepatica*, have been mentioned (see p. 625); however, nothing is known about their formation in the cell. When lipoproteins form extended thin layers, usually they appear to contain bilayers of lipid. For most subunit models the forces that determine their assembly into a membrane are not specified. In others, the ad hoc assumption of a band of hydrophobic binding sites around the circumference of the particle is made. This is hypothetical and no model system exists in which it has been demonstrated.

2. IDENTIFICATION OF SUBUNITS: Two different general approaches can be distinguished in the attempts to demonstrate a subunit structure in membranes. These are morphological methods using electron microscopy and X-ray diffraction techniques to detect structural subunits in intact membranes and disaggregation and fractionation methods attempting to resolve isolated membranes into suspension of small nonmembranous particles, which can then be further characterized by ultracentrifugation, electron microscopy, electrophoresis, chemical analysis, and related techniques. A third approach is largely theoretical; it endeavors to demonstrate that membrane functions can be explained on the basis of cooperative changes in a planar array of subunits (37). The last argument will not be discussed here, because it offers no direct proof for the existence of a subunit structure in membranes. It does not specify the nature of the subunit and would, for instance, be equally applicable to a structure based on a lipid bilayer. Cooperative effects might also be confined to a small number of specific sites, which we do not consider to be incompatible with the bilayer model. There are, of course, phenomena that could most satisfactorily be explained invoking cooperative changes in membrane structure based on conformational changes in subunits. For instance, the observation that colicins can effect profound changes in susceptible bacteria when only one molecule is bound on the surface of the cell (cf. 286) is an often quoted example. An even more attractive case appears to be the postulated contractility of membranes. Unfortunately, the experimental observations on this phenomenon are at present still too few and not decisive enough to warrant a discussion here.

a. Morphological techniques: The evidence that can be derived from electron microscopy of fixed, embedded, and sectioned material, for most membranes, is confined to the demonstration of a globular substructure in the two dense layers of

the unit membrane or, in cases in which dense cross-bridges appear to connect the two layers, confined to a globular substructure comprising the total thickness of the membrane. That caution has to be exercised in the interpretation of such electron micrographs has already been discussed (see p. 625). It should also be pointed out that the observed globular elements usually have diameters of only a small fraction of the section thickness and that it is difficult to explain why they should be observed as rows of single particles in cross-sections.

Negative staining techniques cannot be applied to whole cells but only to disrupted cells and give the best results with isolated membrane fractions. This in itself increases the possibility of artifacts. Such objections do not appear too serious where it can be shown that essential membrane functions have been preserved through the preparation process. However, the artifacts which may arise during the drying of membranes in the negative stain have not been explored. This is especially serious when lipid suspensions are studied as model systems and the structure is deduced only from negatively stained preparations without any corroborative evidence by other techniques, e.g., the globular subunits in the models system of Glauert and Lucy (99, 159; see also 6). Since lipid structures are mainly stabilized through hydrophobic interactions, they must be sensitive to the removal of water during preparation for microscopy. This is clearly indicated by the changes observed in lipid-water systems (161) and natural membranes (85) as the water content is reduced. Moreover, negative staining usually shows only the surface structure of membranes and it is impossible in most cases to determine how far the stain may have penetrated into the membrane.

Shadowing and replication techniques have not been applied very widely in the investigation of membrane structure with the notable exception of freeze-etching. It has already been pointed out that this rather new technique is still plagued by a number of uncertainties in the interpretation of the images obtained. In most cases partial dehydration and/or prefixation of the specimen is used. Which face of the membrane is exposed in a given micrograph is still open to controversy. Consequently, the topographical relation between the observed particles and the membrane is still unresolved and the possibility of artifacts arising during freezing and fracturing or during the shadowing has not been explored sufficiently.

X-ray diffraction has the advantages that it avoids most of the possible artifacts inherent in electron microscopy and that the functional characteristics of the specimen may still be determined after the morphological data have been obtained. Unfortunately, this technique is applicable only to a few sufficiently well-ordered biological structures like the myelin sheath, chloroplasts, and retinal rods. X-ray scattering may yield additional information but has been used very little so far. In some cases, artificial ordering by centrifugation of membrane fractions is possible (86). However, partial drying was found necessary in most instances and this again enhances the possibility of artifacts. Only in the case of retinal rods and chloroplasts has a clear indication of order in the plane of the membrane as an indication of a subunit structure been obtained. This will be discussed below (see pp. 635, 636).

To summarize: The morphological techniques at a level at which the subunit structure of membranes is assumed to occur are not reliable enough to establish clearly that such a structure exists and to analyze it in detail. This objection, however, may not be used summarily when a similar particulate structure can be demonstrated by several different techniques and this has been accomplished for some membranes.

b. Membrane fractionation techniques and reaggregation of membranes: This approach to the identification of subunits implies that inter-subunit bonds are sufficiently different from intra-subunit bonds to allow selective breakage of the former without significantly affecting the latter. Sonication, changes of the ionic environment, and treatment with detergents alone or in combination are the main techniques used. The resulting preparations of "solubilized" membranes may then be further fractionated through differential or density gradient centrifugation, electrophoresis, column chromatography, or by similar means.

Assuming that the starting material is a well characterized and sufficiently pure membrane preparation, three basic problems are encountered in this approach. First, it has to be demonstrated that the "solubilized" preparations obtained are sufficiently homogeneous and contain a sufficiently high proportion of the membrane material so that they could be suspensions of disaggregated

subunits. Secondly, it must be asserted that the membrane simply has not been disrupted into small fragments which may exist either as closed vesicles or as small sheets still retaining the original membrane structure. Such fragments can exist in solutions which are optically almost clear and the fragments may not be sedimentable at rather high g-forces (228). Third, it has to be shown that the particles isolated were actually present in the intact membrane and did not arise through a recombination of components in the disaggregation and fractionation process (e.g., mycoplasma membranes, see p. 634). The pitfalls in characterizing a preparation through ultracentrifugation and electrophoresis are well known and are difficult to overcome even for pure proteins. The same holds true for electron microscopy, which may give values for the size and shape of particles, but makes it difficult to ascertain that a representative sample of the specimen is observed, especially when, as is usually the case, only negative staining techniques are used (70).

Rigorous criteria to prove homogeneity of a specimen with these techniques have not been met in any of the published work on the isolation of membrane subunits. This is somewhat alleviated by the fact that on some preparations a number of different techniques have been used. The second problem, the proof that the isolated particle existed in the intact membrane as a recognizable entity, is even more difficult to solve. Even in the best investigated case, the grana membrane of chloroplasts, the identification of particles observed in the intact membrane with those obtained in the disaggregated membrane preparation has not been achieved.

Often used as an argument for subunit structure is the accomplished reaggregation into membrane-like structures of disaggregated membrane fractions which are supposed to consist of homogenous nonmembranous lipoprotein particle populations. This, of course, is not proof that the particles did exist in the original membrane and a criticism analogous to that discussed for the subunits of assembly still applies.

The rules that govern the assembly of subunits and the geometry of possible structures arising from them have been discussed extensively (34, 35, 134, 136, 209) and have been amply confirmed in cases where subunit structures have been analyzed and where a self-assembly from authentic subunits has been observed (5, 81). Attempts to reassemble membranes usually do not yield the polyhedral or helical structures to be expected from identical subunits but result in the formation of vesicles of widely varying size. To account for these, one would have to relax the specificity of the bonding between subunits and could assume that lipids form rather weak and flexible lateral bonds between the protein subunits and fill the open spaces between them. However, this would interfere with those properties of a subunit structure which were the main reasons for considering it as an alternative to the bilayer structure, namely, self-assembly and cooperative conformational changes. Therefore, subunit models for membranes are usually depicted as consisting of regular arrays of closely packed identical lipoprotein particles and this is difficult to reconcile with the shape and size variations observed.

The structural basis for a subunit is often assumed to be a lipid micelle similar in size to the spherical soap micelles. However, the mixed membrane lipids typically do not form such micelles (66, 218). Instead, if dispersed in large amounts of water, the molecules are found to aggregate into vesicles bounded by bilayers. Binding of protein does not significantly alter the geometry (257, 259). From all model experiments with lipids and proteins, it appears more likely that lipids form a closed bilayer on which the protein aggregates.

3. VARIATIONS OF THE SUBUNIT MODEL: The subunit model has so far been considered mainly as closely analogous to the subunit structure of protein shells in small viruses, substituting lipoproteins for the polypeptides of the virus shells. The size attributed to the lipoprotein subunits of most membranes varies between 40 and 90 A. The fact that all these subunits would have to be identical or that only a few classes of identical particles can be allowed raises a serious difficulty. It would require that the membrane consist of only a few species of lipid and protein molecules, not more than could be packed into a sphere of this diameter. This is incompatible with the variety of different functions found in membranes presumably requiring many different proteins and also possibly requiring specific lipids. Few membranes have been analyzed in detail with regard to their protein composition, but typically they seem to contain a great number of different proteins (e.g., 227). It is unlikely that further investigations will reduce the number

of different proteins found on a membrane; rather, the reverse is to be expected. The lipid composition, which has been better known and known for a longer time, is also found to be rather complex in most membranes and the complexity is increasing continuously as more sophisticated analytical techniques are applied.

This difficulty of accommodating the large number of components in a subunit leads to a modification of the subunit model which postulates that the basic membrane may be composed of only a few predominant lipid and protein species and that all other components are attachments to this membrane but do not significantly contribute to its basic structure. Such a "protomembrane" could, but need not, consist of lipoprotein subunits. It also could consist of a lipid bilayer with protein subunits arranged on its surface.

The lipid composition of many membranes might fit this picture. If the presence in considerable amounts of one or a few structural proteins could be established in a number of different membranes, such a model could become quite attractive. Unfortunately, structural protein which, according to earlier results, could have met these requirements, recently has been discredited as a uniform major component in membranes (see p. 626). If the now well established heterogeneity of the earlier preparations is sufficient to exclude it for such a role, is still difficult to decide. It seems best to postpone further speculations until its origin, the number of polypeptides it contains, the ratios in which they occur with respect to each other and to total membrane protein have been more definitely established. The failure to detect any structural protein in some membranes is certainly a serious argument against a general role in membrane structure.

In this context the lipoprotein envelopes of some viruses are of interest. Sindbis virus has such an envelope which it apparently acquires, as other closely related viruses do, when the nucleoprotein core leaves the host cell (283). The lipid of the envelope is derived from cell lipid and is synthesized before infection. The protein is virus specific and is synthesized after infection (287, 288). The envelope contains only one protein (289, 290). It is very likely that the protein forms a regular array in the virus envelope. The rather constant size and shape of these virus particles is well compatible with a structure determined by subunits; however, the nucleoprotein core could also be the determining factor. Little is known about the functions of the envelopes. If they should serve mainly as a permeability barrier, a lipid bilayer stabilized by protein appears well suited for such a role. However, it seems premature to consider this as a "protomembrane" and an example that would support such a model for the basic structure of cellular membranes. These are certainly more complex and some direct evidence from cellular membranes is required. In spite of the reservations about their general significance for membrane structure, these virus envelopes remain a promising object for further investigation. Especially reconstitution studies would be of interest.

Other models that may be considered variations of the subunit model have been proposed by Kavanau (132) and Lucy (158, 159). They postulate a subunit structure only for the lipid part of the membrane. Both assume that a transition from a bilayer of lipid to a more permeable configuration occurs, with functional changes in membranes. So far there have not been any results from structural studies on membranes that would support these models and they will not be further discussed here.

4. MEMBRANES FOR WHICH A SUBUNIT STRUCTURE HAS BEEN POSTULATED: It does not seem possible, at present, to list the general arguments and evidence for and against the subunit model in the same way as it has been done for the Danielli model. This is due to the fact that the subunit model is not as well defined and that subunit structure has mainly been claimed for some specific membranes without the attempt to generalize to other membranes. Consequently, we shall here examine separately the experimental data for some of the better supported cases. It should be pointed out, however, that most of the observations on these membranes can and have been interpreted on the basis of the classical Danielli model. This will not be discussed extensively. We shall concentrate on the findings and interpretations that are taken as indications for a subunit structure.

a. The inner membrane of mitochondria: This is the most extensively investigated membrane and one on which an attempt at generalization to a subunit model for all membranes has been based. The experimental data and arguments have recently been reviewed in more detail (259), and only

the main conclusions will be summarized here. The well known inner membrane particle, variously called elementary particle, inner membrane subunit, repeating subunit, projecting subunit, etc., has been shown to be identical with the mitochondrial ATPase and, as a single enzyme that can be isolated from and re-bound to the membrane, cannot be identified as a subunit (131). The respiratory chain (cf. 190) may be considered as a functional subunit and might be a structural subunit as well. It must be shown, however, that its components in their proper proportions are clustered together on the membrane. At present a more random distribution where a given enzyme could alternatively react with a number of different molecules of the next component in the chain cannot be excluded (cf. 135) and so far the respiratory chain has neither been identified as a structural subunit in the membrane nor isolated as an intact entity. However, Green and his associates (104) have isolated four lipid and protein containing complexes which can be reconstituted to form a membrane with an apparently intact electron transport chain. These complexes as described meet all criteria for both structural and functional subunits. However, a reinvestigation of the experimental results has raised serious doubts as to the nonmembranous nature of at least one of these complexes (259). It appears that it may consist of fragments of irregular size which still retain membrane structure. This makes it unlikely that in these experiments the membrane was ever broken up into well defined lipoprotein particles and their existence in the intact membrane has certainly not been proven. The reported reaggregation of these complexes into membrane-bounded vesicles has been confirmed for complex IV (cytochrome oxidase). However, the wide variation in size and the frequent appearance of holes in the reconstituted vesicles render their assembly from fully dispersed identical subunits unlikely and rather suggest a reaggregation from membrane fragments of irregular size. Obviously, as long as membrane fragments, which may have retained the basic membrane structure, still exist in such preparations, neither the disaggregation nor the reconstitution experiments can be considered as evidence for a subunit structure.

b. Halobacterium membranes: It has been reported that isolated Halobacterium membranes, when the salt concentration in the suspension medium is sufficiently lowered, disaggregate into a homogenous suspension of particles which comprise the bulk of the membrane material (28). A reinvestigation failed to substantiate this (262). The homogenous particle suspension observed in the earlier work appears to be derived from the cell wall, which in electron micrographs shows a regular array of particles on its surface. The membranes disaggregate into fragments with a rather wide distribution of sizes, the larger fragments still showing a unit membrane structure. If a subunit structure should exist in these membranes, it certainly has not been demonstrated so far.

c. Mycoplasma membranes: Mycoplasma laidlawii, which yields a very clean and easily prepared membrane fraction, has been extensively used in studies of membrane structure. Disaggregation of the membranes through the use of detergent yielded a homogeneously sedimenting material containing most of the lipid and protein of the membrane (215). More recent work, however, demonstrated that the lipid and protein existed as separate particles in these preparations and could be resolved readily in a density gradient (78). When the detergent is removed, the lipid and protein may recombine to form small lipoprotein particles which, under proper conditions, will aggregate into membrane-like structures (77). Missing so far is any evidence that these lipoprotein particles existed in the intact membrane, persist in the reaggregated material, or that their formation is a prerequisite for the reaggregation into membrane-like structures.

Sonication of these membranes under proper conditions gives rise to a much less homogenous suspension of lipoprotein particles. Again, nothing can be said about their preexistence in the intact membrane.

Similar experiments have been carried out with other membranes (e.g., 235). In no case has the investigation been as thorough as with the *Mycoplasma laidlawii* membranes and at least the same criticism applies.

d. Cell junction membranes: A regular structure in the plane of the membrane was first observed in electron micrographs of sections from specialized cell junctions in the goldfish brain (223). It was interpreted as a subunit-like structure confined to the outer surface of the cell membrane, which still showed the unit membrane appearance in cross-section. Later observations on a similar

structure in sectioned heart muscle cell junctions confirmed this interpretation (220). When the tissue was fixed and stained with routine techniques, two parallel unit membranes were seen at the site of the junction separated by a gap of 20 A. When a lanthanum salt was added to the fixation and dehydration media, this gap was filled by an extremely electron-dense material. In tissue so treated, sections parallel to the plane of the junction showed a hexagonal pattern which was not observed in the routinely fixed and stained material. Freeze-etching shows a comparable pattern (O. Kreutziger. Personal communication). Similar structures were seen in other cell junctions using freeze-etching (S. Bullivant. Personal communication) and recently a reasonably pure fraction of such cell junctions has been obtained from liver cells where negative staining could be used to demonstrate again a very similar surface pattern (15).

This seems to be the first case in which a regular surface pattern can be demonstrated in specialized surface areas for a wide variety of cell membranes and in which comparable pictures are obtained with quite different electron microscopic techniques. The evidence so far indicates that this pattern is confined to the outer surface of the cell membrane and it does not exclude a basic bilayer structure. Some evidence for a special functional role of this part of the cell membrane in the direct transmission of electrical impulses and intercellular communication is beginning to accumulate (30, 156, 157). The isolation of these junctions from liver cells promises rapid, further progress in the elucidation of their structure.

e. Photosynthetic membranes: The problems that have arisen in studies on chloroplast membranes are very similar to those encountered in mitochondrial membranes. The photosynthetic unit, first postulated by Emerson and Arnold (76; cf. 25, 203) can be considered a functional subunit. The morphological studies have yielded more data on the structure in the plane of the membrane than are available for mitochondria, and not only electron microscopy but also X-ray diffraction and polarized light studies have contributed valuable information. In spite of this the structure is still heavily disputed and no functional membrane fragment has been isolated that would approach the size of the subunits deduced from the morphological studies.

Early polarized light studies of chloroplast membranes indicated form and intrinsic birefringence attributable to lipid and protein and a structure of alternating lipid and protein lamellae was proposed (94, 95, 171). A granular structure on the surface of the membrane was first observed by Steinmann (253) in electron micrographs of shadowed preparations. Park (204–206) extended these observations and showed that a regular square array of these particles did occur in some areas of spinach chloroplast membranes. The particles are $\sim$ 200 A in diameter and may be composed of four 90 A subparticles. Park also postulated that the 200 A particles constituted the (sub)units of photosynthesis and coined the term "quantasome." Originally, the quantasomes were thought to be located on the inner surface of the membrane. Further results using lipid extraction and freeze-etching, which showed additional membrane faces with a different but also particulate texture, led to a modification of the earlier interpretations. This resulted in a model of membrane structure for the chloroplasts that does not specify the exact position and conformation of lipid and protein but describes the quantasome as embedded in a lipid "matrix" only a small part of which may have bilayer structure. Essentially, the model postulates a subunit structure for chloroplast membranes (25, 26, 207).

Mühlethaler (186, 187), on the basis of essentially identical morphological observations, also obtained mainly by freeze-etching, came to a different conclusion. He interprets these observations on the basis of the Danielli model and assumes that the observed particles are multienzyme complexes which are located on the surface of a lipid bilayer and partially penetrate into the lipid. This interpretation appears to be in reasonable agreement with observations by Moudrianakis et al (cf. 181, 182) who used mainly negative staining and fractionation techniques. These authors do not propose a detailed membrane model of their own, but sharply disagree with Park's interpretation of a subunit structure and the identification of the quantasomes as the photosynthetic unit or part of the electron transport chain. They assume that the particles seen by Park are similar to the ATPase or inner membrane particles of mitochondrial membranes in their function and relation to the photosynthetic membrane.

Still another model is proposed by Menke

(172, 173) and Kreutz (139–143) who, in addition to electron microscopical observations, make extensive use of X-ray diffraction data from dried and wet chloroplasts and also from whole leaves. Their model contains a continuous layer of lipid only one molecule thick but with hydrophilic groups on both surfaces and a protein layer only on one surface of this lipid bilayer. The protein layer is assumed to consist of subunits. This model has been extended also to the photosynthetic membranes of algae and bacteria for which a particulate structure in the plane of the membrane has been deduced both from electron micrographs and from X-ray diffraction patterns (173, 174). The structure of the protein layer in chloroplast membranes has been further detailed by using X-ray data and studies of isolated chloroplast proteins (124, 144). The pattern deduced appears compatible with the electron micrographs of shadowed and freeze-etched preparations but the proposed model differs considerably from those of Mühlethaler or Park and Branton.

Still another entirely different model has been developed by Benson (18, 280). It features subunits composed of protein and lipid, in which the hydrophobic portions of the lipids penetrate deeply into the core of protein particles. The morphological evidence for this model is weak, based on the granular appearance of the membranes in stained and sectioned material. Mainly chemical data and theoretical considerations are used to support it, which results in a very detailed and highly speculative structure.

Considering the available data, it becomes obvious that a particulate structure in the plane of the photosynthetic membranes very likely exists. For this a considerable amount of independent evidence also has been accumulated by investigators who did not propose detailed models (98, 123, 146). The exact distribution of lipids and proteins within the membrane is still a matter of controversy and a subunit structure for this membrane in the sense of repeating lipoprotein particles cannot be considered established. However, it mav be worthwhile to point out that the Menke and Kreutz model constitutes an extreme case of the variations possible in the Danielli model. Here the lipid bilayer is only one molecule thick, the asymmetry of the membrane is very high because only one protein layer is present, and this protein layer has a regular particulate structure.

f. Photoreceptor membranes of the eye: The early work on fine structure has been reviewed by Moody (178). Results of polarization and electron microscopy appeared compatible with a bilayer model and the membranes in rod and cone outer segments as well as in the rhabdomeres could be shown to develop from the cell membrane. In fact, the early polarized light studies of Schmidt (236) already indicated a structure very similar to myelin and were subsequently considered as another demonstration of the widespread occurrence of lipoprotein bilayers. Fernández-Morán (79) reported a globular substructure in the disc membranes of outer segments. However, his electron micrographs have been criticized because the technique he used introduced a strong granularity of the same size as the subunits superimposed on the whole picture (245). Nilsson (191, 192 195), avoiding this objection by the use of a slightly different technique, obtained pictures in which the membranes seemed to consist of light 25 A globules with a center-to-center spacing of 50 A. These observations, however, are open to the same criticism that applies to similar electron micrographs of mitochondrial and other membranes (see p. 625).

Quite different particles and spacings were observed in a study of the rod outer segments with X-ray diffraction and negative staining (19). In dried membranes a square array of 40 A particles with a unit cell dimension of 70 A was found in the plane of the membrane. The particles may be rhodopsin, because antibodies to rhodopsin are bound to the membranes in a square array of the same dimensions, which can be observed in negatively stained preparations. They show the square pattern much more clearly than similar preparations which have not been treated with antibody (67, 282).

Unfixed, moist membranes fail to show the well defined square array of particles. The number of nearest neighbors, which is four in the dried membrane, in moist membranes varies with the temperature from 3.8 at 4.5° C to 2.4 at 42.5° C (20, 21). The particles are apparently in a fluid environment that permits translations in the plane of the membrane under physiological conditions. Neither electron microscopy nor X-ray diffraction can decide whether the particles are located on the surface of the membrane or extend

through the thickness of the membrane. The situation here is similar to that encountered in the study of photosynthetic membranes and the conclusions with regard to the question of a subunit versus bilayer structure at present are the same, except that the suggested mobility of the particles in the plane of the membrane argues more strongly against a subunit structure.

g. Sarcoplasmic reticulum: The sarcoplasmic reticulum represents another membrane for which a subunit structure may appear likely from functional considerations. It is known to contain a calcium transport system which may occupy a substantial part of its total membrane area (71, 111, 112, 167); 40 A particles have been observed in negatively stained preparations. They are connected by a short stalk to the outer surface of isolated sarcoplasmic reticulum vesicles and resemble the inner membrane particles of mitochondria, but are considerably smaller (126, 127). Similar particles have been seen on endoplasmic reticulum membranes from other sources (16, 162). They have, of course, been called subunits (168). So far no relationship to the calcium transport system has been established for these particles and their function is unknown. Since they are not removed by lipid extraction but disappear upon trypsin treatment, they may consist at least partly of protein.

Martonosi (169) has reported "solubilization" of the isolated sarcoplasmic reticulum membranes by bile salt treatment and reconstitution of vesicles with ATPase activity and active Ca^{++}-transport from the solubilized material. These experiments cannot be interpreted as evidence for a subunit structure because the same criticism leveled against the "solubilization" of mitochondrial and Mycoplasma membranes applies here. Neither has the nonmembranous nature and homogeneity of the "solubilized" material been established nor has a separation of lipid and protein in the solubilized form been excluded. In fact, Martonosi has produced some evidence that such a separation does occur in the solubilization process.

These data obviously do not provide sufficient reason to consider seriously a subunit structure for this membrane; however, in an electron microscope study Hasselbach and Elfvin (111) have demonstrated, by coupling a sulfhydryl group (SH-group) reagent to ferritin, that a rather dense population of SH-groups exists on the outer surface of isolated sarcoplasmic vesicles. They could further show, by selective protection and blocking, that these groups may belong to a component of the calcium transport mechanism. Indications of a regular arrangement were also obtained.

The calcium transport mechanism appears to be the predominant functional subunit of these membranes. Should it occur as a fixed complex with the density of distribution indicated by the ferritin-labeling experiments, a situation similar to that in mitochondrial and chloroplast membranes would exist. There is, however, abundant evidence for the unit membrane appearance of the sarcoplasmic reticulum and spin-labeling studies show the existence of a low viscosity hydrocarbon region in these membranes (125). This is difficult to reconcile with a membrane consisting of lipoprotein particles. The best interpretation at present seems to be that the sarcoplasmic reticulum membranes contain a lipid bilayer and may have a particulate structure mainly confined to the protein moiety; however, the available evidence clearly is insufficient to reach any firm conclusions.

5. GENERAL EVALUATION OF THE SUBUNIT MODEL: We have tried to define the subunit model in general terms. Our criticism, obviously, applies only within the limits of this definition. We think, however, that the definition given here is conceived broadly enough and it should include all proposed versions of the model that are found in the literature.

As we have seen, in no case can the subunit structure of a membrane be considered to have been established beyond reasonable doubt, at least not in the sense of a structure composed primarily of identical lipoprotein particles. The most suggestive evidence for a subunit structure has been obtained from the grana membranes of chloroplasts, the disc membranes of the retinal rods and certain cell junction membranes. In the retinal rods and in the cell junctions, it appears likely that the particle structure is confined largely to the protein layer of the membrane; in the case of chloroplasts, at least one group of investigators interprets the data in a similar way. If these interpretations are correct, the data are not incompatible with the bilayer model as we have defined it here. Such a model would then appear as a further development in which mainly the structure in the protein layers has been specified, and in which membranes or membrane

areas with one dominating function may well show a repeat structure.

The main objection to the subunit model is that the principal features of a subunit construction have not been found in membranes and appear difficult to reconcile with their general properties. Membranes are not uniform in their composition. A self-assembly from one or a few classes of identical building blocks has not been demonstrated. In fact, the rather limited number of different shapes and the restrictions in their size that are characteristic for a subunit structure appear incompatible with the wide variety in shape and size of membranes found in cells or in isolated preparations. It would also appear very difficult to explain the growth and differentiation of membranes with the development of new functions in existing membranes on the basis of a general subunit model. Some principles of subunit structure could be applicable to small, specialized areas in a membrane, such as cell junctions. However, this remains to be demonstrated and the subunit model, in our opinion, has so far contributed very little to an understanding of general membrane structure and function.

IV. CONCLUSIONS

We have attempted to review the data and arguments that usually are advanced for the major models of membrane structure and to evaluate these objectively. Some bias in the representation may be unavoidable for anyone working in the field. Nevertheless, we shall attempt a synthesis of the material presented, rather than be content to point out how inconclusive most of the evidence still is. The picture that emerges is vague necessarily, but may be of some heuristic value.

We have concluded that the evidence seems generally to favor the Danielli or bilayer model as it has been defined in section II. However, this model fails to provide structural principles that could be used to explain the differences in the function of different membranes. Little was known about the functional characteristics of lipid bilayers before Mueller and Rudin and their collaborators (184) developed the black lipid films as a model for biological membranes. More data were soon added by other investigators using the same or similar systems (266). From their results it now appears possible to conclude that a lipid bilayer could constitute the main passive permeability barrier for cells and possibly also for cell organelles. It forms stable partitions between aqueous compartments and has a thickness, electrical properties, permeability to water and many other solutes, which are close to the values for the same parameters observed in biological membranes. It also has been shown that some of the specific characteristics of natural membranes, notably the selective high permeability to some small ions, may be duplicated in the model membranes by the addition of proteins or peptides and related compounds. The modifications thus introduced comprise only a small fraction of the total membrane area, leaving the lipid bilayer as the general permeability barrier largely intact. It seems reasonable to assume that cellular membranes could be built according to the same principle. The lipid bilayer would constitute the basic permeability barrier, which is modified according to the specific requirements of the cell or cell organelle. The protein layers of the Danielli model, in addition to stabilizing the lipid bilayer, may be expected to be the main sites and agents for these required modifications largely because their known diversity could meet the wide variety of required functions. Differences in lipid composition, of course, also contribute to the functional characteristics of different membranes. It has, for instance, been suggested that cholesterol regulates permeability through its effect on the fluidity of the hydrocarbon chain layer (43), and in model systems sphingomyelin was found a necessary component for the simulation of action potentials (183). That some lipids may have specific functions, such as cardiolipin in mitochondria or the linolenic acid-containing galactolipids of chloroplasts, is suggested mainly by their consistent occurrence in these organelles and by reactivation experiments on lipid-depleted structures (18, 88). Whether they participate directly in these functions or through their effects on protein is not known, and their participation may be quite unrelated to their role in forming a bilayer. The direct evidence so far implicates lipids mainly as modifiers of passive permeability (62, 63, 164). This has also been demonstrated in model systems, e.g., the influence of cholesterol on water permeability (87). In general the observed variability in the lipid composition of membranes with changes of diet or other environmental conditions (e.g. 43, 51, 117, 214, 216, 285)

argues for a lesser role of the lipid in specific functions.

At special sites the protein may reach deeply into the lipid phase or penetrate to the opposite surface as, for instance, the experiments on lipid extracted mitochondria suggest, or the experiments with black lipid films, in which the modifying activity of proteins adsorbed from one side is abolished by proteases added to the other side. At such sites the bilayer structure would then be interrupted but no alternative structure can presently be specified. If repetitive, closely packed, identical sites occur, they could be formally described as subunits. However, if very many structurally and functionally different sites exist in a given membrane, the subunit concept would no longer be applicable. Even with closely packed identical sites, it still remains to be shown that the structural principles of subunit construction are operative in such membranes or membrane areas. In most instances in which we can estimate the number of specific sites and the part of the total area which they occupy, both appear to be small. The only model relevant to the structure of membranes at present then appears to be the bilayer model. Acceptable models that would help us understand the structure of sites carrying out specific functions so far do not exist. Their development would seem to be the next logical step in membrane research.

The general model presented appears reasonable from another independent point of view. The genesis of the earliest cells from nonliving precursors would require a boundary structure not readily soluble in water which could confine the first interacting and replicating prebiotic molecules. This would allow the interior composition to be independent to some degree from accidental changes in the environment and would allow significant concentrations of identical molecules to accumulate. Since the probability that such a boundary structure has arisen as a single covalently bonded unit is vanishingly small, it was probably formed through the assembly of smaller molecules. The rather accidental formation of the first protenoid molecules makes it unlikely that enough identical molecules could have been present in high enough concentration to form such a structure from protenoid subunits in a self-assembly process. Also, the properties of such a hypothetical protenoid subunit shell to serve as a stable permeability barrier appear questionable. Polar lipid molecules, however, even when they are not identical will readily associate into uniform thin layers forming closed structures. Such vesicles appear to have the required properties, especially if they are further stabilized by the adsorption of protein on their surfaces. Goldacre (101) has pointed out that lipid or lipoprotein films which form spontaneously at the air-water interface collapse under pressure from wind or other forces to form closed structures bounded by lipoprotein bilayers which enclose the aqueous subphase. He has postulated that this constitutes an essential step in the genesis of the first cell. While the formation of a surface film is not a necessary condition for the formation of lipoprotein bilayer-bounded vesicles, it overcomes the difficulty of explaining how sufficient concentrations of lipid and protein could have been attained in the environment presently considered the most likely for the evolution of living cells. In any case, lipids would appear to be the logical choice for the primary structure-forming component of biomembranes. In the further development suitable specific modifications could have been introduced into such primary bilayer membranes. The variability, both in conformation and in composition, would render a bilayer membrane an eminently suitable component for the development of living cells. It is difficult to see how a subunit membrane could acquire a similar flexibility.

Thermodynamically stable structures are required for the earliest cells or cell precursors. Therefore, a spontaneous formation from their components should be demonstrable, as is the case for the bilayer membranes. This does not mean that cells still use this mechanism. With the introduction of many different specific functional sites, an organism acquiring a special mechanism for the replication of its membrane would have a genetic advantage that would probably allow it to outgrow the more primitive cells. In all cases that have been studied, the insertion of new material into a pre-existing membrane appears to be the mechanism used for the synthesis of new membrane. The one possible exception known, the synthesis of vaccinia membrane in infected cells, is worth further study, but even here no indication of an assembly from lipoprotein subunits has been found (50).

If the validity of the arguments advanced here is granted, the question arises about how extensive the modifications in any given membrane are; in

other words, how much of the bilayer structure still may be preserved. The observations on natural membranes, which support the Danielli model, seem to indicate that most of the total area in many membranes may still have a recognizable bilayer structure. However, the amount of bilayer may vary considerably from one membrane to another. A priori one would expect little deviation in structures like the myelin sheath, which appears to function mainly as a permeability barrier, and would expect extensive deviations in the chloroplast and mitochondrial membranes, which are highly specialized in functions quite different from those of the basic permeability barrier. Obviously, the question can only be answered by a detailed study of different membranes.

Fortunately, new techniques are now being applied to membranes which may yield the necessary data. Spin-labeling studies with lipid soluble probes, for instance, are one promising approach to the problems discussed. They allow some estimate of the amount and "fluidity" of hydrocarbon regions in membranes. With this technique, hydrophobic regions of low viscosity have already been found in isolated membranes of the sarcoplasmic reticulum and in more complex systems. The failure to detect such regions in mitochondria is noteworthy (125, 133, 145). Similar conclusions have been drawn from NMR spectra and calorimetric data of these membranes (249). Calorimetric measurements show phase transitions in isolated membranes very similar to those taking place in lipid bilayers (249, 251). These data as well as those from ORD, CD and infrared spectra are still difficult to interpret in complex particulate systems, especially since they have been applied before simple model systems were sufficiently investigated, but eventually they may provide data on the configuration of the constituting molecules and the environment of reactive groups (43). This will facilitate the construction of models for a specific membrane from direct evidence. Only after a number of well-supported models for different membranes have been worked out will it be possible to decide how representative a general membrane model actually can be. Another possible approach to the same problem is the detailed study of model membranes which have been modified to simulate specific functions of natural membranes.

In summary, the bilayer model still appears to approximate best the general structural principles found in biological membranes. We depict it as a continuous bilayer of lipids with a disordered central hydrocarbon region. Protein is predominantly arranged on the surface of the lipid. Its amount and configuration may vary. Both ionic and hydrophobic bonding between protein and lipid stabilize the structure. These interactions can occur without extensive penetration of the protein through the lipid layer. Deviations from this main structural principle are likely to be found in all membranes and will vary with the different functional requirements. The observations commonly taken as evidence for a subunit structure in some functionally highly specialized membranes or areas of membranes are not incompatible with this concept. There is, however, no evidence so far that the structural principles governing subunit assemblies contribute significantly to membrane structure in general. Whether new general structural principles for specific functional sites in membranes will emerge remains to be seen.

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