Bioenergetics (1971) **2**, 233–247

On the Structure of Biological Membranes: The Double-Tiered Pattern

T. Wakabayashi, E. F. Korman and D. E. Green
Institute for Enzyme Research, University of Wisconson, Madison, Wisconsin 53706

(*Received 3 August 1971*)

Abstract

A set of six biological membranes has been examined electron microscopically in positively-stained sections at both low and high resolution. At low resolution, all six membranes exhibit a "railroad track" pattern, while at high resolution all six membranes exhibit a pattern of a double-tier of globular "particles". The correspondence between the railroad track pattern and the double-tiered pattern is considered. The relationship of the double-tiered pattern to the bimodal properties of the constituent protein and phospholipid molecules is discussed.

Introduction

For some time, workers in the field of biological membrane structure have been aware of the "bilayer" character of biological membranes. The bilayer nature of the lipid of such membranes has been studied by a wide variety of techniques and is widely recognized. In fact, the bilayer nature of membrane lipid is so well accepted that lipid bilayer has been assigned a central structural role in the unit membrane model of biological membrane structure in the form of a bimolecular leaflet. However, the arrangement of membrane proteins within the biological membrane structure has been a good deal less clear. Stoeckenius and Engelman have given a critical review of these matters.[1]

In an effort to study biological membrane structure, as far back as 1960 Fernandez-Moran observed a double-tiered pattern of globular "particles" in the membranes of the mitochondria of retinal rods examined at high resolution in electron microscopy.[2] Sjöstrand has for some time insisted on the globular nature of the components of biological membranes[3] and has adduced a large number of electron micrographs in support of this thesis. These earlier observations made a minimal impact on the theory of biological membrane structure since, although the then widely accepted unit membrane model could accommodate proteins which would exist as or appear as globular particles in electron microscopy at high resolution, it was not so easy to see how the bilayer lipid inherent in the model could give rise to globular particles. Also, it was not at all clear how the four-layered structure of the unit membrane model, consisting of a bilayer of lipid "sandwiched" between two layers of protein, could give rise to a double-tiered pattern.

Recently, in this laboratory we have studied a set of six biological membranes of diverse types which have been positively stained, sectioned, and examined electron microscopically at both low and high resolution. In all cases we have observed a railroad track pattern at low resolution, while at high resolution we have observed a characteristic double-tiered pattern of globular particles. From these and the earlier findings, we have deduced a general concept concerning a double-tiered arrangement of globular proteins in these, and possibly other, biological membranes.

Experimental

Procedures for Electron Microscopy

Fixation. Samples were fixed for 1 h at 0° by exposure to a solution which was 1% in glutaraldehyde, 0·05 M in cacodylate buffer of pH 7·4 and 0·25 M in sucrose, and then washed in a medium 0·25 M in sucrose and 0·05 M in cacodylate buffer of pH 7·4. After washing, the samples were exposed for 1 h at 0° to a solution which was 1% in osmium tetroxide, 0·05 M in cacodylate buffer of pH 7·4, and 0·25 M in sucrose.

Dehydration. After interaction with osmium tetroxide, the samples were exposed first to 25% ethanol which was 1% in uranyl acetate, and then to solutions of gradually increasing ethanol concentrations. Exposure to 100% ethanol was repeated three times. For complete dehydration, the samples were exposed twice to absolute propylene oxide, each exposure lasting for 10 minutes.

Embedding. The dehydrated samples were exposed first for 20 minutes to a mixture of equal parts by volume of propylene oxide and Epon, and then embedded by interaction with 100% Epon for four hours.[4]

Electron microscopy. The embedded specimens were sectioned with a diamond knife, and then examined in a Hitachi HU-11B or HU-11E electron microscope operated at 75 or 100 KV.

Preparation of membranes. Beef heart mitochondrial suspensions were prepared by the method of Crane, Glenn and Green;[5] suspensions of red blood cell ghosts from beef blood by the method of Marchesi and Palade[6] as modified by Penniston and Green;[7] suspensions of sarcoplasmic reticulum from rabbit skeletal muscle by the method of MacLennan;[8] myelin suspensions from beef brain by the method of Murdock *et al.*[9] and Autilio *et al.*[10] as modified by R. Zand of the University of Michigan in Ann Arbor (private communication); spinach chloroplast suspensions by the method of Rocha and Ting;[11] lipid depleted heavy beef heart mitochondria by the method of Fleischer *et al.*;[12, 13] and micellar dispersions of Asolection by the method of Fleischer *et al.*[14]

Results

The double-tiered pattern in electron micrographs of membranes of different function and from different sources. We have examined five different membranes in positively stained thin sections both at low and high resolution. These electron micrographs are shown in Figs. 1–4 respectively. Through the kindness and permission of Drs. Stanley W. Watson and Charles Remsen of the Woods Hole Oceanographic Institute we are also including in this study a high resolution electron micrograph of the mesosomal membranes of a marine bacterium cell (*Nitrocystis oceanus*) shown in Fig. 5. All six of the membranes

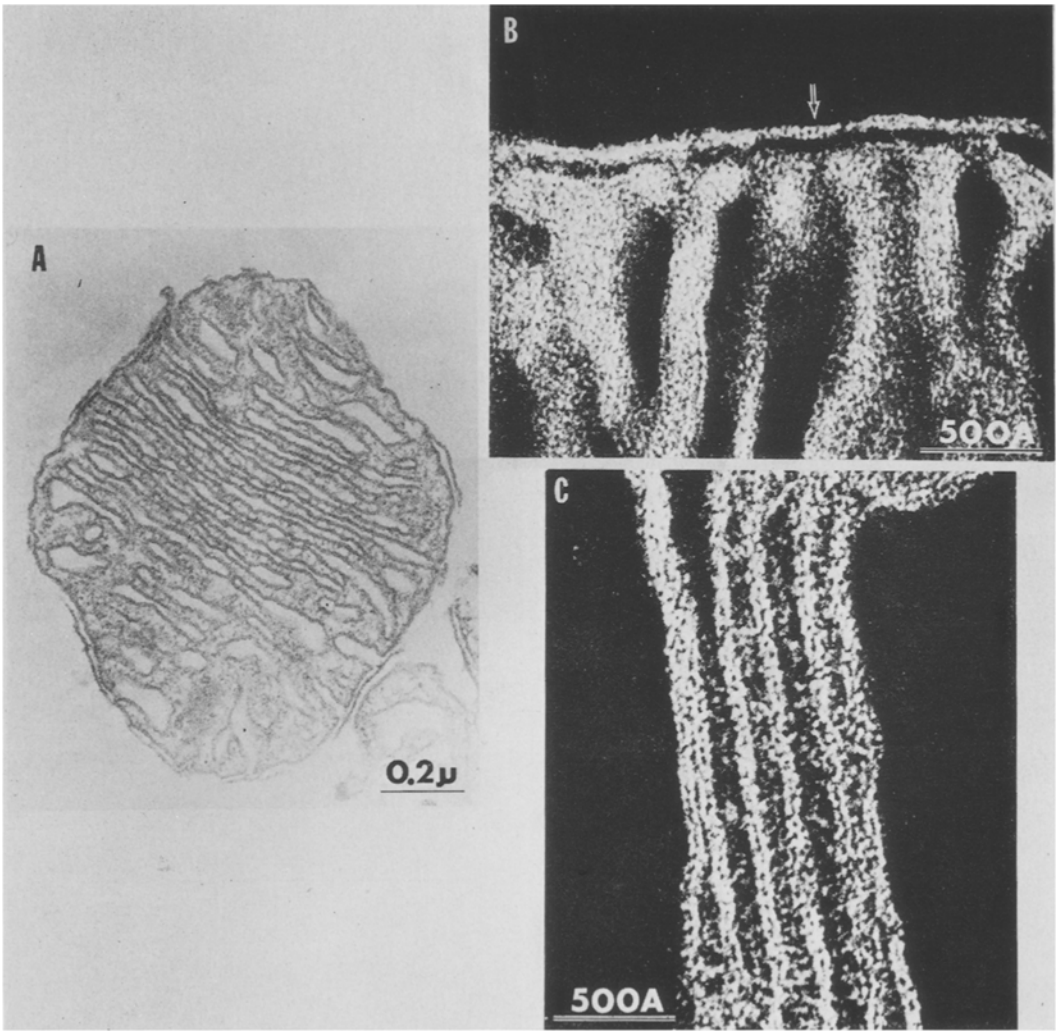

Figure 1. Electron micrographs of mitochondria. A. Whole mitochondria at low magnification. (×175,000); B. Segment of outer membrane at high magnification. ($\times 1 \times 10^6$); C. Segments of inner membrane at high magnification ($\times 1 \times 10^6$).

examined showed the railroad track pattern (two dark lines separated by a space) at low resolution, and a double-tiered pattern of globular particles at high resolution. The clarity and definition with which the double-tiered pattern of globular particles can be seen varies, but the double-tiered pattern of these membranes is unmistakable. The double-tiered pattern of globular particles is unambiguously and consistently demonstrable in the mitochondrial inner membrane (Fig. 1C), the red blood cell ghost membrane (Fig. 2B), the membrane of the sarcoplasmic reticulum (Fig. 3B), and the mesosomal membranes of the marine bacterium (Fig. 5).

We have also demonstrated the railroad track pattern at low resolution and the double-tiered pattern of globular particles at high resolution for the mitochondrial outer

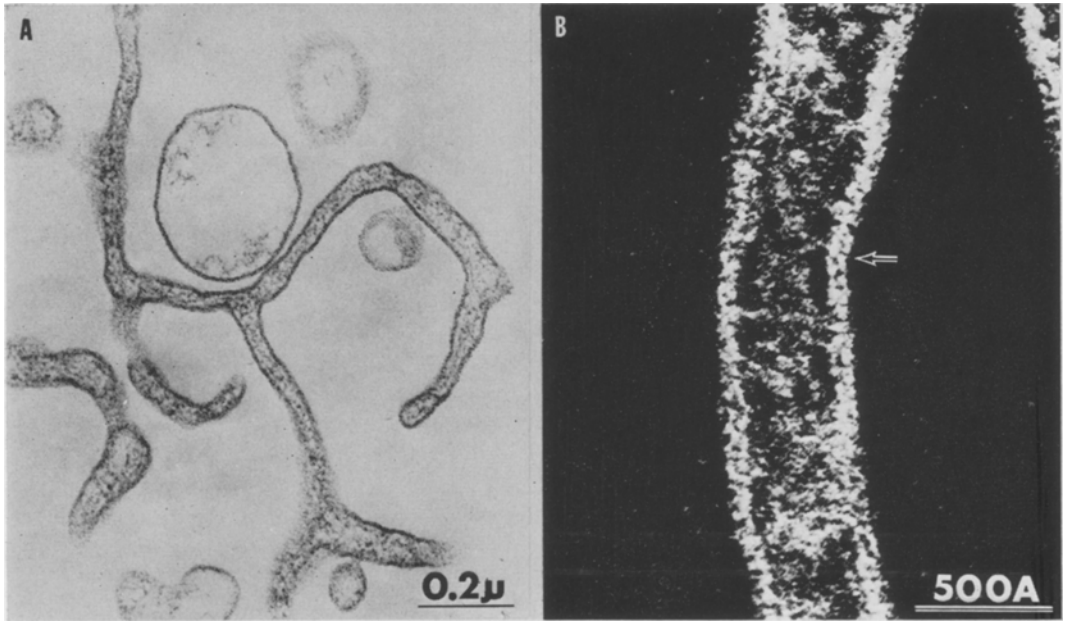

Figure 2. Electron micrographs of red blood cell ghosts. A. Low magnification. (×175,000); B. High magnification. ($\times 1 \times 10^6$).

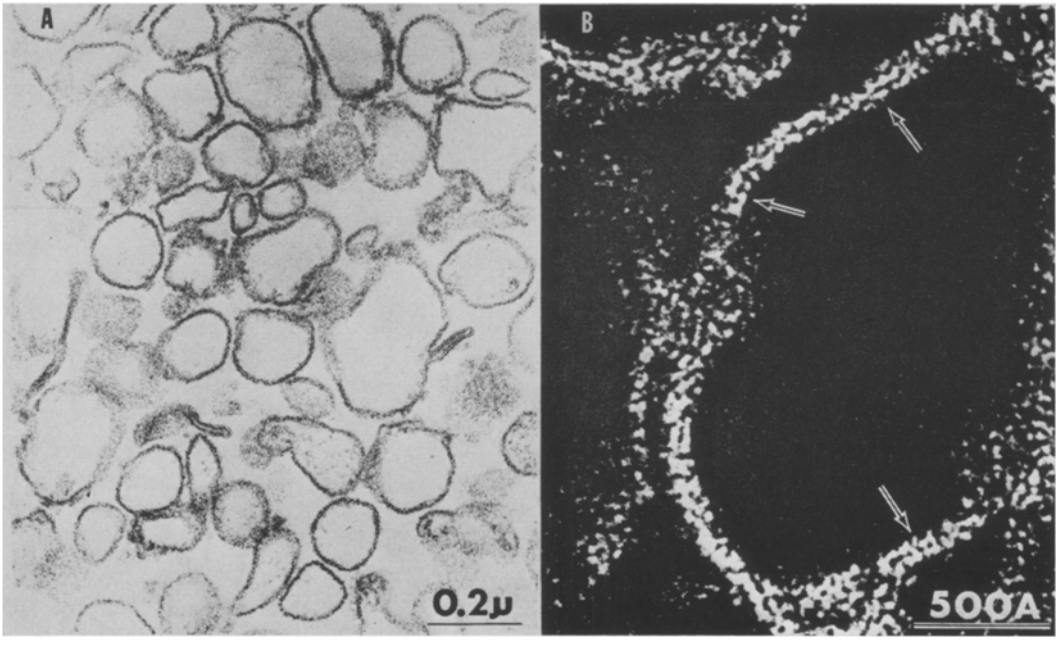

Figure 3. Electron micrographs of sarcoplasmic reticulum. A. Low magnification. (×175,000); B. High magnification. ($\times 1 \times 10^6$).

membrane (Fig. 1B) and for the lamellar membrane of spinach chloroplasts (Fig. 4), but the results with these latter two membranes have been somewhat less satisfactory. This is not to say that we find patterns other than the railroad track or double-tier of globular particles in these membranes. The problem posed by these membranes is not that they show patterns different from other membranes, but rather that regions within these membranes appear "jumbled". Nevertheless, despite the rather high incidence of jumble in these membranes, when a clear pattern in the structure of these membranes *is* recognizable at high resolution, it is always the double-tiered pattern of globular particles. Also, the pattern in high resolution electron micrographs of lipid-extracted mitochondrial inner membrane (Fig. 6) is generally hard to discern clearly, although in the same membrane the double-tiered pattern of globular particles is very easily and consistently demonstrated prior to lipid extraction. The point to be made by the high resolution electron micrographs in Figs. 1–6 is that, despite experimental difficulties, all these different membranes show at least *some* regions with the double-tiered pattern of globular particles, and what is equally important, they exhibit *no other* pattern. Solvent extraction of phospholipid from a membrane seems to accentuate jumbling, but here again, despite the jumbling, it is apparent that the railroad track pattern at low resolution and the double-tier of globular particles at high resolution are still demonstrable, as they were in the original un-extracted membrane.

High resolution electron micrographs of micellar dispersions of phospholipid. We have used for this study micellar dispersions of Asolectin—a commercial perparation containing the phospholipids of soybeans. By means of sonication, the phospholipid is dispersed in the

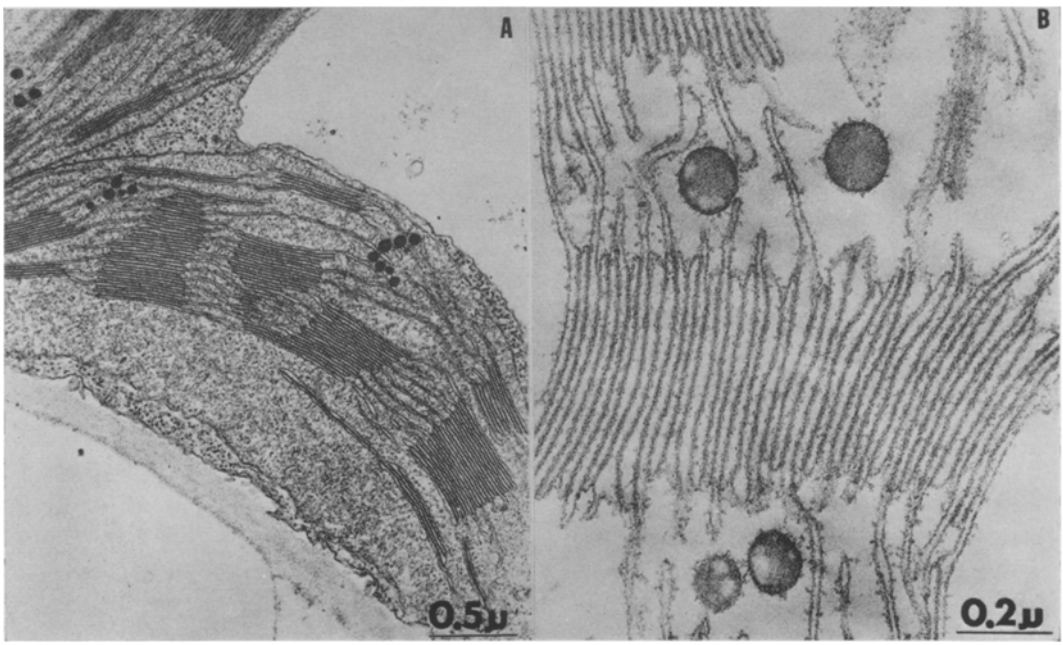

Figure 4a and 4b.

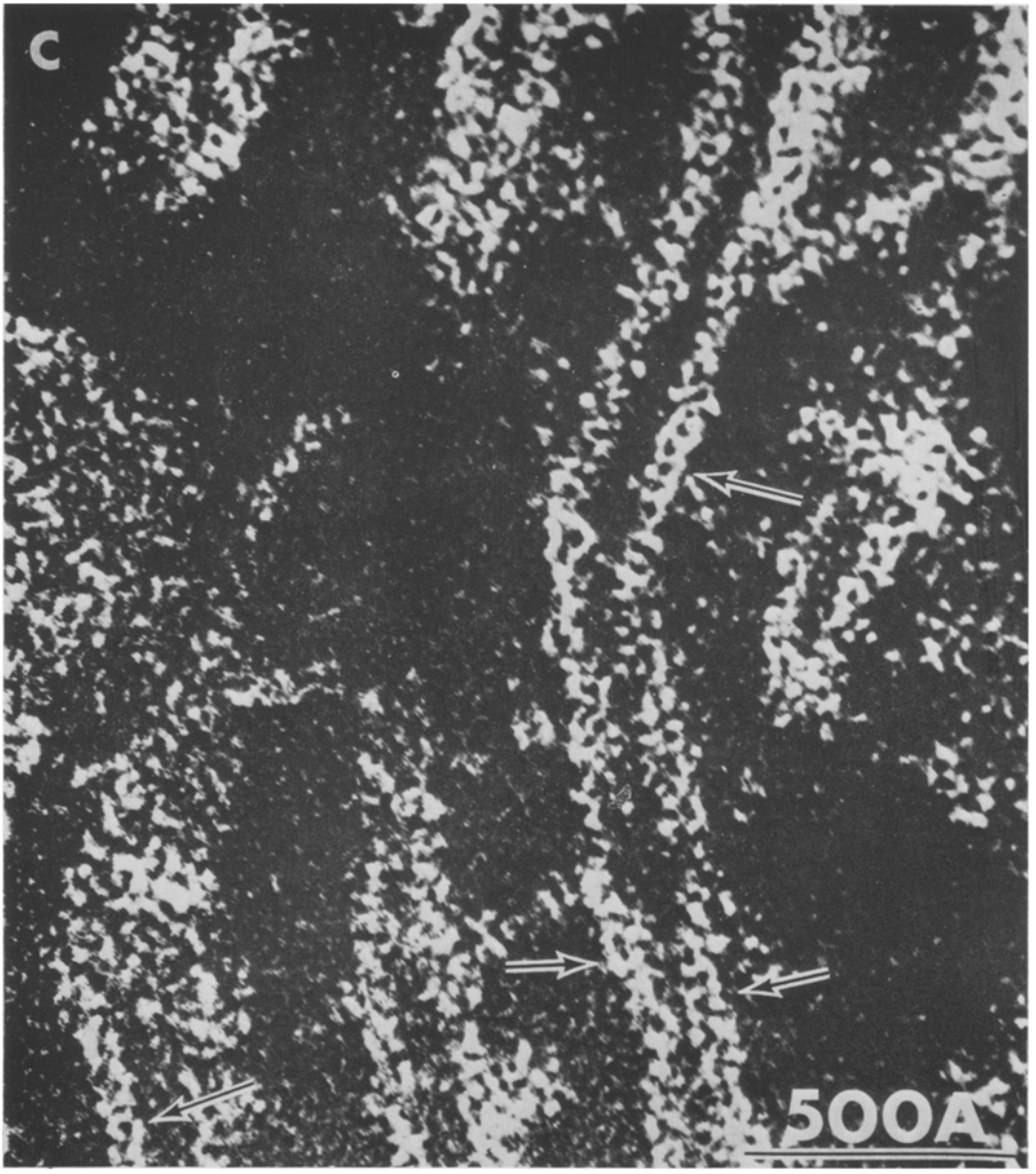

Figure 4. Electron micrographs of chloroplasts. A. Low magnification showing general appearance of chloroplast. (×6,500); B. Low magnification showing fushion of lamellae and granae. (×175,000); C. High magnification of the lamella membrane. ($\times 1 \times 10^6$).

form of myelin-like structures which are built up of layer after layer of bilayer phospholipid wrapped like paper on a roll. The unit of construction of the micellar structure is thus a phospholipid bilayer. The experimental question we posed was how such a laminar arrangement of bilayer structures would show up in electron micrographs using the same

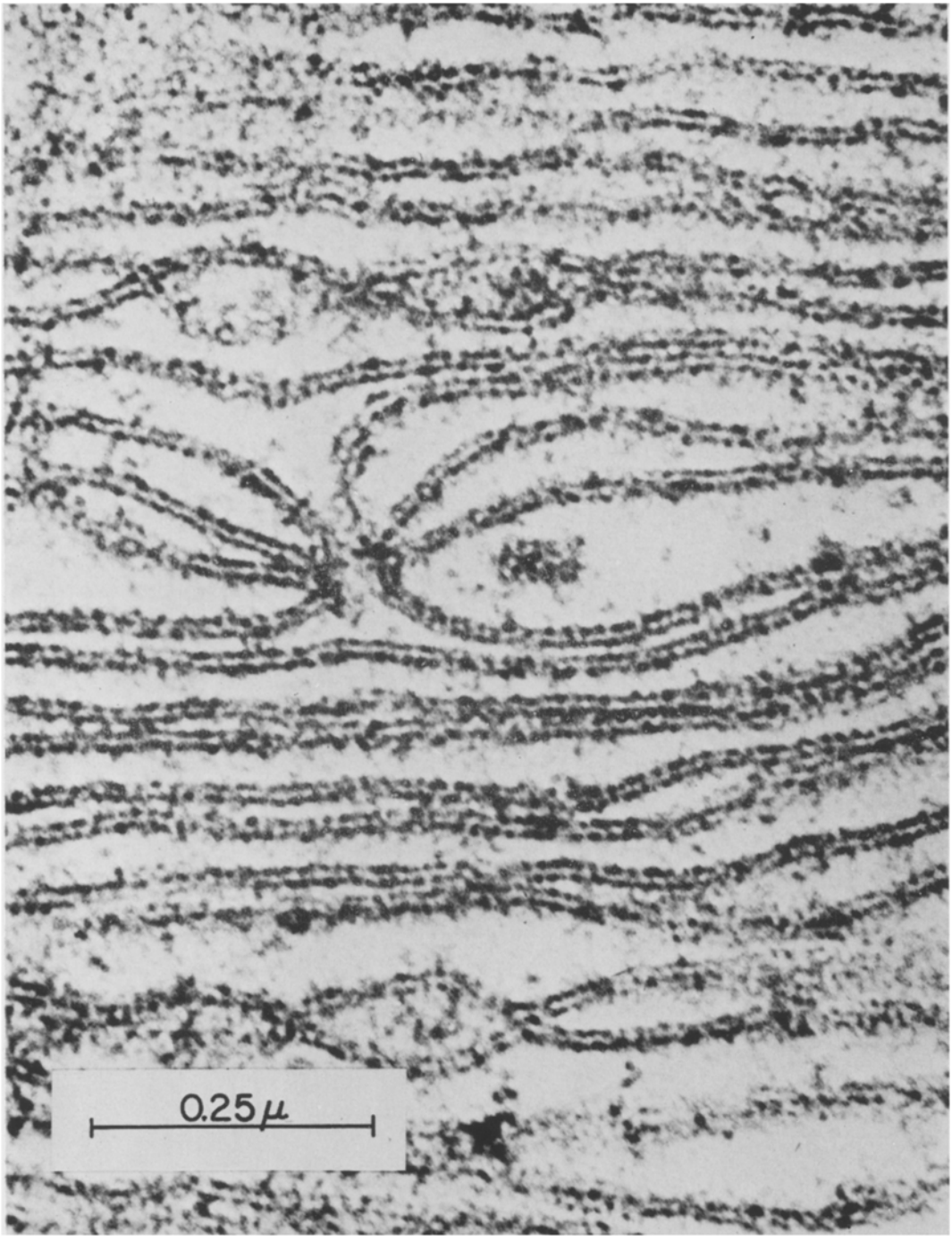

Figure 5. Electron micrograph of positively-stained thin section of *Nitrocystis oceanus*.

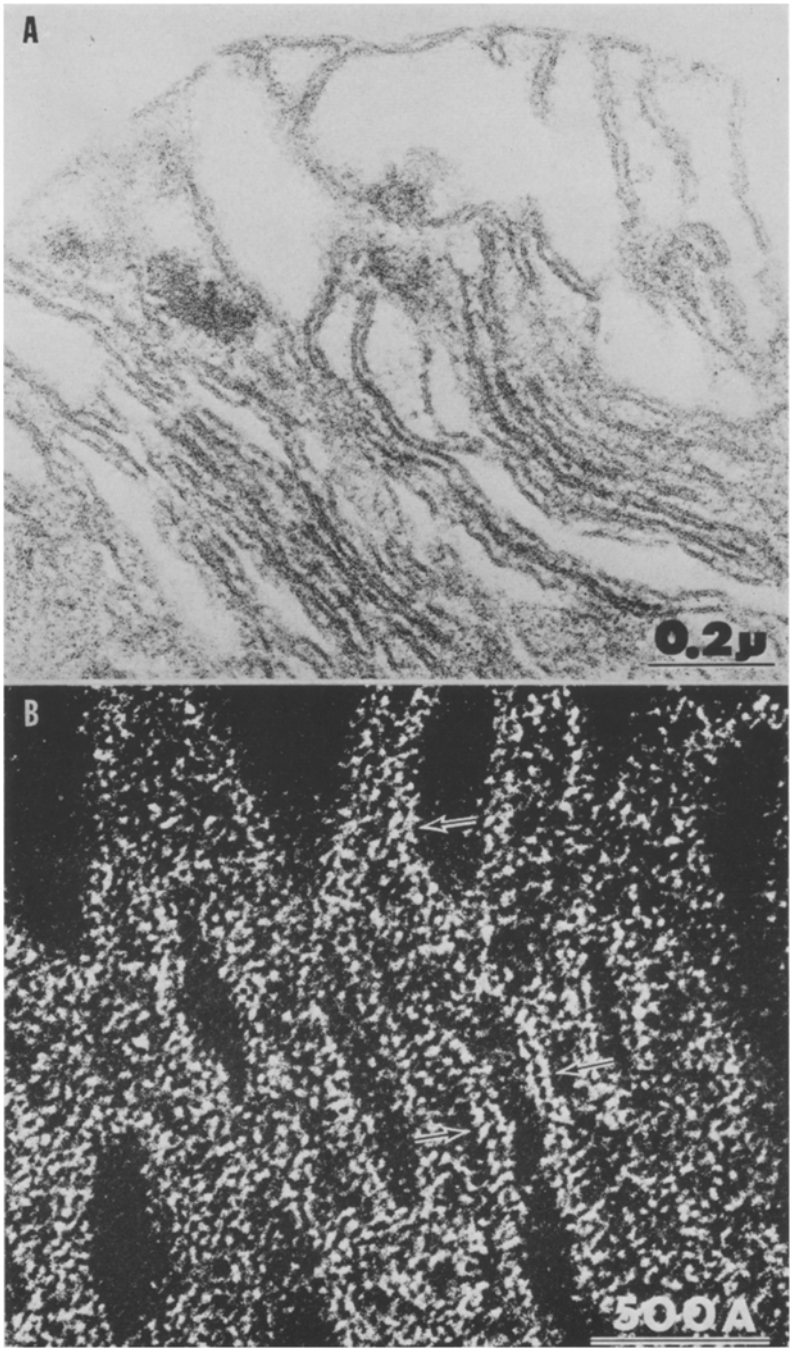

Figure 6. Electron micrographs of solvent extracted mitochondria. A. Low magnification. (×175,000); B. High magnification. (×1 × 10^6).

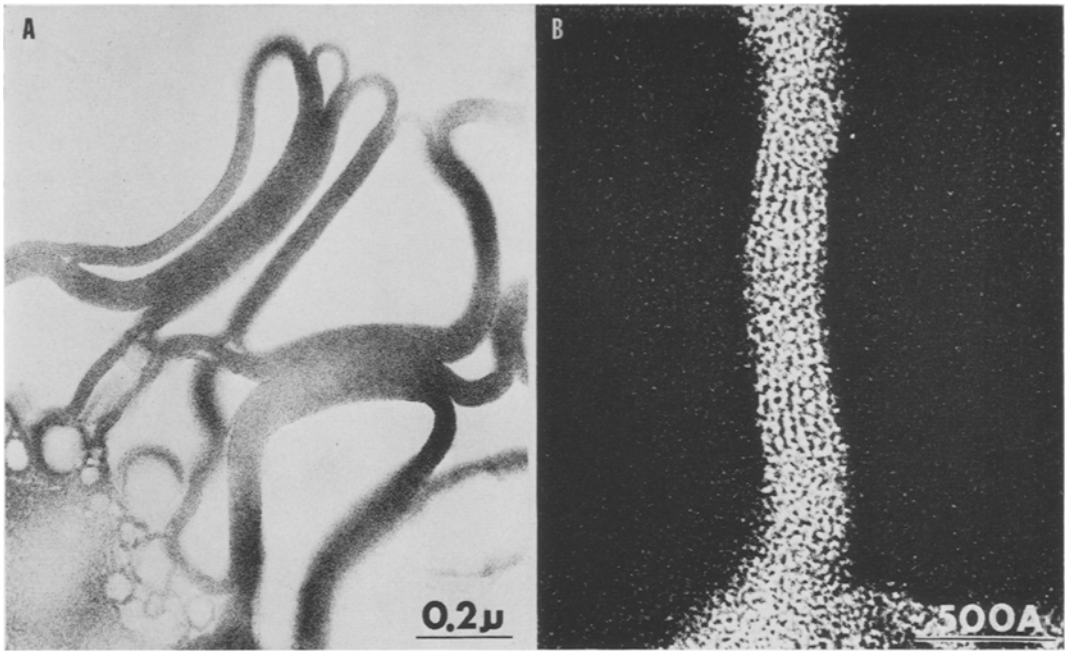

Figure 7. Electron micrographs of a positively stained thin section of a dispersion of Asolectin. A. Low magnification. (×175,000); B. High magnification. ($\times 1 \times 10^6$).

procedure for the preparation of the specimen of micellar phospholipid as was used in the preparation of membrane specimens. Figure 7 shows one typical electron micrograph. We see row after row of globular particles each about 32 Å in diameter.

Size of the globular particles in electron micrographs of membranes. Table I provides a summary of the average diameters of the globular particles in each of four mammalian membranes. The averaged diameters are based on a large number of measurements and vary from 25 Å to 29 Å. The thickness of the membranes are also given.

TABLE I. Thickness of various membranes and the diameters of their globular particles

Membrane	Thickness (Å)	Diameter of globular particles (Å)
Outer mitochondrial membrane	67 ± 3	25 ± 4
Inner mitochondrial membrane	68 ± 3	25 ± 4
Inner mitochondrial membrane (lipid extracted)	68 ± 4	23 ± 4
Red blood cell ghost	75 ± 4	29 ± 3
Sarcoplasmic reticulum	74 ± 6	27 ± 3

Discussion

The most striking observation made in this study is the occurrence of the double-tiered pattern of globular particles in the electron micrographs of diverse biological membranes at high resolution. This striking pattern might be considered as an artefact, and thus

not related to the original membrane structure, or it could be considered as a meaningful pattern related to the original structure. We adopt the latter view, and believe that the pattern requires explanation. The unit membrane model of biological membrane structure is not able to provide an explanation for such a pattern. The membranes which have been studied here, which at high resolution exhibit the double-tiered pattern of particles, exhibit a railroad track pattern at low resolution. The correlation observed here in several functionally quite different membranes between the railroad track pattern and the double-tier of globular particles may have very important implications for the generality of the double-tiered pattern of globular particles in biological membranes. There is a vast literature which already documents the widespread occurrence of the railroad track pattern in biological membranes.[15] Thus, although the studies cited here are limited to a small number of membranes, because of their diversity in type, we would like nevertheless to suggest strongly that the correlation between the railroad track pattern at low resolution and the double-tiered pattern of globular particles at high resolution may be general for biological membranes.

If, indeed, the double-tiered pattern of globular particles observed at high resolution in the electron micrographs of these biological membranes is general for biological membranes, then it is extremely important to consider that pattern in terms of its two main characteristics, namely the globularity of its particles, and its double-tiered pattern. Does the globularity of the particles in the stained specimens reflect a structural globularity in the original membrane? What is the chemical identity of the globular particles? What is the relationship of the double-tiered pattern of globular particles to the structure of the original membrane?

The answers to the first two of these questions are inter-related. It is quite clear that both lipid (in the absence of protein) as well as membrane proteins (from which lipid has been removed by solvent extraction) can each independently give rise to globular particles in positively stained specimens examined electron microscopically at high resolution. Thus, in biological membranes, both the lipid and protein components very probably contribute to the globular particles seen electron microscopically. Any one particular globular particle visualized could thus be either protein or lipid.

Since many of the globular particles visualized in microscopy are protein in nature, a very important question which arises is whether or not the known molecular sizes of membrane proteins are compatible with the observed diameters of the globular particles. The globular particles observed in the high resolution electron micrographs of membranes in this study are fairly uniform in size and all lie within the range of 25–31 Å. While it is not possible to make precise calculations of protein molecular weights from these dimensions without rather precise information about the geometric asymmetry of such particles, given some degree of asymmetry and using the cubic relationship between the average radius of a protein particle and its molecular weight, the observed diameters would not be incompatible with molecular weights in the range of 1×10^4 to 5×10^4 Daltons. While there are reports in the literature of membrane proteins with molecular weights either smaller than 10,000[16] or larger than 50,000[17], the majority of membrane proteins thus far studied fall within the limits specified above. There is, therefore, at present no real basis for concern that the globular particle sizes observed by high resolution electron microscopy and the particle sizes predicted from known molecular weights of isolated membrane proteins are not in accord. A recent theoretical

study by Gates and Fisher[18] has led those authors to the conclusion "that the maximum average thickness of globular proteins should be 30 Å to 40 Å". Moreover, the diameters would be less for other shapes, and since protein molecules are not likely to be perfect spheres, the observed diameters will in general be less than the predicted maximum. These considerations reinforce our confidence that the 25 Å to 30 Å range that we have observed for diameters of the globular particles in the double-tiered patterns is not out of line with the range of known molecular weights of membrane proteins.

One of the questions raised earlier was "Does the globularity of the particles in the stained specimens reflect a structural globularity in the original membrane?" Of the two types of molecular species in the biological membrane which could give rise to globular particles, namely protein and lipid, the lipid very likely does not exist in a globular state in the original untreated membrane, but very likely exists rather in a sheet-like bilayer. Thus, it is probably only the protein which is at all likely to exist in a globular form in the original untreated membrane. However, even in the case of the protein, it has not been shown that it exists as globular entities in the original untreated membrane. The globularity of the particles seen in the electron micrographs of biological membranes, of which many particles are protein in nature, could be due to an original globularity of those proteins in the original untreated membrane, or could be due to a globularity induced by the interaction of non-globular proteins with osmium tetroxide or with some other material used in the fixing and embedding procedures.

While it is easy to see how protein molecules could exist within a biological membrane as globular particles or as structures which, upon interaction with osmium tetroxide, would give rise to globular particles, it is rather more difficult to see how bilayer lipid, either isolated or within the membrane, could give rise to such globular particles. However, preparation of specimens for electron microscopy subjects the membranes to a dehydration procedure in which they are exposed to ethanolic solutions of progressively decreasing water content. When the water content of the medium falls below a critical level, phase inversion of the lipid molecules could take place and globular lipid particles be formed. This type of event may also take place with Asolectin phospholipid fixed with oxmium tetroxide, with the only difference being that in Asolectin the phase transition is not modulated by the presence of proteins.

In the absence of any kind of prior knowledge about biological membranes other than the fact that they are built up of molecules (of lipid and globular proteins whose chemical properties are here purposely left unspecified), there is no *a priori* reason why the arrangement of molecules could not be completely random and show no ordered structure whatsoever, but instead be merely a jumble of molecules. Indeed, we have observed a high incidence of jumble in the high resolution electron micrographs of mitochondrial outer membrane (Fig. 1B), of the lamellar membrane of the spinach chloroplasts (Fig. 4C), and of lipid-extracted mitochondrial outer membrane (Fig. 6). It was pointed out above that in these membranes, when a pattern does occur which is recognizable in the midst of extensive jumble, the pattern is always that of a double-tier of globular particles. Equally important is the fact that *no other* pattern is ever observed. This complexity, namely the occurrence of a unique pattern of globular particles arranged in a double-tier observed in the midst of jumble, may arise because we are observing not only the membrane continuum, but rather a more complex system of proteins which includes the membrane continuum as only one if its constituents. In the case of the mitochondrial

outer membrane, it has been shown that the membrane can be markedly depleted of enzymatic activity,[19, 20] and when isolated exhibits one dominant protein band in gel electrophoresis[21, 22] while in electron micrographs it appears in the form of vesicular membranes[22]. These findings strongly suggest that the mitochondrial outer membrane probably has a single type of protein species which is *intrinsic* to the membrane continuum and which gives rise to the double-tiered pattern of globular particles, while the membrane continuum has associated with it an array of *extrinsic* proteins which give rise to the jumble.

Given that biological membranes have an ordered arrangement of their intrinsic molecules, and given that the ordered arrangement involves a layer or layers of globular protein molecules (analogous to the well-documented laminar arrangement of membrane phospholipid molecules), there could conceivably be one, two, three, or indeed any number of layers of intrinsic proteins. Such various possible laminar structures could give rise to patterns having any one of a number of layers or tiers of protein and lipid in high resolution electron micrographs. A double-tiered pattern is therefore not the only possible one for biological membranes to exhibit in high resolution electron micrographs. In fact, we have observed biological membranes which appear in high resolution electron micrographs to be triple-tiers of globular particles.[23] The reality of the triple-tiered pattern of globular particles is undeniable, but it can be shown that all such triple-tiered patterns of globular particles arise by the "fusion" of *two* biological membranes, each individual membrane of which exhibits a double-tiered pattern of globular particles. The point we wish to develop here is that the double-tiered pattern of globular particles should be recognized as unique. We would also like to point out that a double-tiered pattern of globular particles is not the pattern to be anticipated from the unit membrane model of biological membrane structure, as will be discussed in detail below.

The fact that a biological membrane from which lipid has been essentially completely removed by appropriate solvent extraction exhibits the same thickness (see Table 1) and the same double-tiered pattern of globular particles as does the unextracted membrane provides evidence that the intrinsic membrane proteins are involved in determining biological membrane structure. Thus, the central structural role assigned to bilayer lipid in the unit membrane model of biological membrane structure, essentially to the exclusion of membrane protein as a determinant of biological membrane structure, cannot be correct. On the other hand, it is not our thesis that membrane proteins have a structural role to the exclusion of membrane lipid. Rather, it is our thesis that the membrane proteins and bilayer lipid *both* are determinants of the structure of biological membranes, and that both contribute to the double-tiered pattern of globular particles seen in high resolution electron micrographs of such membranes. This implies that the membrane proteins are probably arranged within the biological membrane as a double-tier of 25–30 Å-diameter globular units (or units which upon interaction with osmium tetroxide assume such globular form), while the lipid is in the membrane in the form of a bilayer (bimolecular leaflet) which upon interaction with osmium tetroxide undergoes a phase inversion giving rise to globular sets of phospholipid molecules.

On the basis of the observed diameters of the globular particles seen in the high resolution electron micrographs of osmium tetroxide-fixed sections of Asolectin micelles, where the globular particles measure 30–32 Å in diameter, it has been estimated that one such particle could represent a set of about 10 phospholipid molecules. Since the globular particles in this material are more or less uniform in size, the phase inversion

from bilayer to globular particles is probably a quite orderly process involving approximately the same number of phospholipid molecules for each particle formed. Thus, it is very likely that when the bilayer lipid within a biological membrane undergoes this phase inversion, it may very well do so with the same orderliness, and indeed may form a double-tier of globular particles which reflects the original double-tiered character of the arrangement of the phospholipid molecules in the lipid bilayer within the membrane. Moreover, the phase inversion from bilayer to a double-tier of globular particles could be facilitated by the phase inversion occurring within a biological membrane with its double-tier of protein molecules.

According to these suggestions, in which proteins which may be globular give rise to globular particles in electron micrographs of sections of positively-stained biological membranes at high resolution and in which membrane bilayer lipid may give rise to globular particles of sets of phospholipids molecules arranged in a double-tier reflecting the double-tiered nature of the bilayer, it would be predicted that the unit membrane model of biological membrane structure should exhibit a *four*-tiered pattern in such electron micrographs. That is, the lipid bilayer "backbone" of the unit membrane model could give rise to a double-tier of globular particles of sets of phospholipid molecules, while the two layers of globular proteins on the two surfaces of the lipid bilayer could each give rise to a layer of globular particles, to give a final four-tiered pattern in high resolution electron microscopy. Even if the lipid bilayer backbone were to give rise to only a single layer of globular particles, the unit membrane model of biological membrane structure would predict at the very least a three-tiered pattern of globular particles. The facts that a four-tiered pattern has *never* been seen, and that three-tiered patterns arise only by the fusion of two membranes, as discussed earlier above, make the unit membrane model of biological membrane structure extremely unlikely, if judged only on the basis of these considerations.

The arrangement of a double-tier of proteins, which may be globular, and of a lipid bilayer, into an integrated structure strongly implies certain properties are inherent in the protein units. If a double-tier of globular protein units exists *as such* in the biological membrane, and does so in conjunction with a lipid bilayer whose individual phospholipid molecules are known to be bimodal, it is strongly implied that the protein units would also be *bimodal*. In a fashion which is analogous to the arrangement of bimodal phospholipid molecules in the lipid bilayer (having hydrophobic fatty acid chains internal and thus screened from the aqueous medium by the polar heads which are external) in which the bimolecular leaflet structure is quite clearly an expression of the associative properties of bimodal phospholipid molecules in an aqueous medium, we deduce that a double-tiered pattern of globular particles of protein would be an expression of the associative properties of bimodal globular protein molecules in an aqueous medium (probably having hydrophobic regions towards the center or interior of the double-tier and polar regions on the two surfaces which face into the aqueous medium). Both bimodal phospholipid molecules and bimodal protein molecules can enter into a common structure (probably having a spacial coincidence of their polar and non-polar regions) in which each contributes to and determines the structure, and by doing so, together decrease the energy of the entire system below that for bimodal phospholipid in water (unassociated with protein) in the presence of bimodal globular protein in water (unassociated with lipid).

The bimodality of membrane proteins which may be globular is central to our thesis. The bimodality of membrane proteins discussed here is also inherent in the Vanderkooi-Green protein-crystal model of biological membrane structure.[24,25] The infra-red spectroscopy studies of Wallach and Steim[26] and the optical rotary dispersion studies of Lenard and Singer[27] show that protein in membranes contain more alpha-helix conformation than in aqueous medium, indicating that membrane proteins have regions which are in a hydrophobic medium within the framework of the membrane structure, and these proteins very likely are bimodal in nature. Furthermore, Colacicco predicted and demonstrated that proteins derived from biological membranes possess unique properties which differentiate them from soluble proteins such as ribonuclease and lysozyme.[28] Bimodal membrane proteins penetrate lipid monolayers on water surfaces much more rapidly than do soluble proteins to become incorporated into the lipid films and to orient at the water interface with a large part of their structure in the alpha-helical conformation within the hydrophobic regions of the lipid film. The bimodal properties of membrane proteins are probably ultimately responsible for the dramatic effects which such membrane proteins have upon the surface tension and surface pressure properties of lipid monolayers.[28,29]

What has emerged from the study of the electron micrographs of sectioned and positively stained materials viewed at high resolution is a picture consistent with a membrane with bimodal protein molecules which may be globular and bimodal phospholipid molecules. The bimodal character of the proteins is expressed in terms of the associative properties of those molecules to give a double-tiered structural arrangement. The bimodal character of the phospholipid molecules is expressed in terms of the associative properties of those molecules to give a double-tiered structural arrangement, namely a phospholipid bilayer. The structural integrity of the biological membrane is not attributable exclusively to either protein or lipid, but its stability is due rather to the unique circumstances of both protein and phospholipid expressing their respective bimodal properties simultaneously within a single structural framework.

Acknowledgements

We are grateful to Dr. Robert Zand of the University of Michigan for samples of purified myelin, to Dr. Jenny Smoly for the preparation of micellar asolectin, and to Mrs. June E. Sundquist for chloroplast preparations. The skilled technical assistance of Miss Ghousia Ghatala and Miss Nancy Lee with the electron microscopic studies, and of Mr. Donald R. Silver with the photography, is gratefully acknowledged. We also wish to thank Dr. Garret Vanderkooi and Dr. Giuseppi Colacicco for many helpful discussions. This investigation was supported in part by Program Project Grant GM-12847 from the National Institute of General Medical Sciences (USPHS).

References

1. W. Stoeckenius and D. M. Engelman, *J. Cell. Biol.*, **42** (1969) 613.
2. H. Fernandez-Moran, *Circulation*, **26** (1962) 1039.
3. F. S. Sjöstrand, *Nature*, **199** (1963) 1262.
4. J. D. Luft, *J. Biophys. Biochem. Cytol.*, **9** (1961) 409.
5. F. L. Crane, J. L. Glenn and D. E. Green, *Biochim. Biophys., Acta* **22** (1956) 475.
6. V. T. Marchesi and G. E. Palade, *J. Cell. Biol.*, **35** (1967) 385.
7. J. T. Penniston and D. E. Green, *Arch. Biochem. Biophys.*, **128** (1968) 339.
8. D. H. MacLennan, *J. Biol. Chem.*, **245** (1970) 4508.
9. D. D. Murdock, E. Katona, and M. A. Moscarello, *Canad. J. Biochem.*, **47** (1969) 818.
10. L. A. Autilio, W. T. Norton and R. D. Terry, *J. Neurochem.*, **11** (1964) 17.

11. V. Rocha and I. P. Ting, *Arch. Biochem. Biophys.*, **140** (1970) 398.
12. S. Fleischer and B. Fleischer, *Methods in Enzymol.*, **10** (1967) 406.
13. S. Fleischer, B. Fleischer and W. Stoeckenius, *J. Cell. Biol.*, **32** (1967) 193.
14. S. Fleischer, G. Brierley, H. Klouwen and D. E. Slautterback, *J. Biol. Chem.*, **237** (1962) 3264.
15. J. D. Robertson in *Intracellular Transport* (K. B. Warren ed.) Academic Press, Inc., New York (1966) p. 1.
16. M. T. Laico, E. I. Rouslahte, D. S. Papermaster and W. S. Dreyer, *Proc. Nat. Acad. Sci. (U.S.)*, **67** (1970) 120.
17. Y. Hatefi, K. A. Davis and W. G. Hanstein, *Biophysical Society Abstracts, 15th Annual Meeting*, New Orleans, (1971) 119a.
18. R. E. Gates and H. F. Fisher, *Proc. Nat. Acad. Sci. (U.S.)*, in press, 1971.
19. E. Racker and H. Proctor, *Biochem. Biophys. Res. Commun.*, **39** (1970) 1120.
20. T. Olivecrona and L. Oreland, *Biochemistry*, **10** (1971) 332.
21. J. Smoly, T. Wakabayashi, A. D. F. Addink and D. E. Green, *Arch. Biochem. Biophys.*, **143** (1971) 6.
22. W. Neupert, and G. D. Ludwig, *Eur. J. Biochem.*, **19** (1971) 523.
23. T. Wakabayashi, R. F. Brucker and D. E. Green, in preparation.
24. G. Vanderkooi and D. E. Green, *Proc. Nat. Acad. Sci. (U.S.)*, **66** (1970) 615.
25. G. Vanderkooi and M. Sundaralingam, *Proc. Nat. Acad. Sci. (U.S.)*, **67** (1970) 233.
26. D. Chapman, *Biological Membranes-Physical Fact and Function*, Academic Press, New York, New York (1968).
27. J. Lenard and S. J. Singer, *Proc. Nat. Acad. Sci. (U.S.)*, **56** (1966) 1828.
28. G. Colacicco, *J. Colloid Interface Sci.*, **29** (1969) 345.
29. G. Colacicco, Conference on Membrane Structure, *Annals N.Y. Acad. Sci.*, in press, 1972.