X-RAY DIFFRACTION PATTERN FROM A BILAYER WITH PROTEIN OUTSIDE

ALLEN E. BLAUROCK

From the Cardiovascular Research Institute, University of California San Francisco, California 94122

ABSTRACT The X-ray diffraction pattern from a lipid bilayer has been reported previously; a series of fairly regularly spaced bands was both predicted and observed. In this note it is predicted that adding protein molecules at one or both surfaces of the bilayer will give rise to a cross-interference effect. For smaller amounts of protein, a more or less obvious ripple will be introduced into the bilayer pattern. The amount of protein, its thickness, and the distance from the bilayer to the protein layer all can be readily estimated from an observed ripple. Deciding whether the protein is all on one side or else distributed on both sides of the bilayer may be more difficult; by carefully recording and measuring the intensity near the center of the pattern one may be able to distinguish between the two possibilities. For larger amounts of protein, there will be more profound changes in the diffraction pattern. The theory developed here is applied in the following paper to a lipid dispersion incubated with cytochrome *c* and will be applied in a subsequent paper to a bacterial envelope. In an appendix it is shown that the patterns reported previously for several natural membranes do not confirm prediction for a normal, continuous lipid bilayer with all the protein outside. Thus it is doubtful that a structure of this kind is valid for these membranes.

INTRODUCTION

The profile of electron density characteristic of the lipid bilayer in cross section includes two peaks, corresponding to electron-dense head groups at the surfaces (Fig. 4 *a*). There is a broad trough between the peaks since the layer of liquid-like fatty chains is less electron dense than water.

The X-ray diffraction pattern predicted (Wilkins et al., 1971) for a uniform bilayer, dispersed as single sheets in water, is a series of fairly regularly spaced bands of X-ray intensity (B^2 in Fig. 4 *b*). The Bragg's law spacings[1] of the centers of the

[1] The term "Bragg's law spacing" is used in a formal sense; a periodic (e.g., multilayer) structure is not implied. When periodic structure is absent the diffraction is said to be continuous. In this case, the diffraction angle for any point on the diffraction pattern is used to calculate the corresponding Bragg's law spacing (see Eqs. 1 and following).

bands are approximately D/h, $h = 1, 2, 3, \ldots$, where D is the center-to-center distance between the peaks in the profile. The bilayer diffraction pattern has been inferred (Rand and Luzzati, 1968; Levine and Wilkins, 1971) and observed (Wilkins et al., 1971) for some lipid-water mixtures, thus confirming bilayer structure.

In contrast, lipid vesicles incubated with cytochrome *c* give a low-angle X-ray diffraction pattern of a rather different form: a higher-frequency ripple is added onto the broad bilayer bands (Blaurock, 1973). A similar effect is observed for the envelope of *Halobacterium halobium* (Blaurock, Oesterhelt, and Stoeckenius, manuscript in preparation). In both cases the likely structure is a bilayer (lipid bilayer or plasma membrane) with protein molecules (cytochrome *c* or cell wall) in a layer outside.

In this note it is shown that cross interference between a bilayer and protein molecules at the surface will introduce a ripple into the bilayer X-ray diffraction pattern. The theory is applied to the lipid-cytochrome *c* membrane in the following paper and will be applied to the bacterial envelope in a subsequent paper.

THE PREDICTED DIFFRACTION PATTERN

For membranes uniform in the plane the X-ray diffraction pattern will be proportional to the square of the Fourier transform of the electron density profile (Wilkins et al., 1971). The Fourier transform of the electron density profile for a bilayer membrane in water is denoted by $B(K)$; the general form of $|B(K)|^2$ is illustrated in Figs. 1, 2, and 4. K is defined by

$$K = 4 \cdot \pi \cdot \sin(\theta/2)/\lambda, \tag{1}$$

where θ is the diffraction angle and λ is the X-ray wavelength. The Bragg's law spacing corresponding to a given θ is equal to $2 \cdot \pi/K$.

The Fourier transform for a uniform layer of protein in water is denoted by $P(K)$. As indicated in the Discussion, the general form of $|P(K)|^2$ will be a large band centered at the origin ($K = 0$) and smaller subsidiary bands; Figs. 1, 2, and 4 illustrate the form.

The bilayer and the protein layer are assumed parallel to one another and spaced a distance S apart, center to center (Figs. 1–4). The two parts will give rise to a cross-interference effect in the profile diffraction pattern, as follows.

For simplicity it is first assumed that both profiles are symmetric and that B and P are the respective transforms when the structures are centered at the origin; both B and P are then real valued. The square of the Fourier transform of the compound profile can be expressed in terms of B and P:

$$B(K)^2 + P(K)^2 + 2 \cdot B \cdot P \cdot \cos(K \cdot S); \tag{2}$$

The diffraction pattern, e.g. photographic film density, will be proportional to ex-

pression 2. At the end of this section the corresponding expression is given for the case when either the bilayer or the protein layer is asymmetric.

The first two positive terms in expression 2, $B(K)^2$ and $P(K)^2$, are for diffraction as though from the bilayer alone and the layer of protein alone, respectively. The third is the term for cross interference:

$$2 \cdot B(K) \cdot P(K) \cdot \cos (K \cdot S); \tag{3}$$

This term oscillates between positive and negative values and therefore corresponds to ripples in the diffraction pattern. The frequency of the ripples is proportional to S. Figs. 1–4 illustrate expression 2.

Fig. 1 *a* shows a simplified bilayer profile and, to the right, the simplified profile for a layer of globular protein molecules. The layer of 30 Å-diameter protein molecules is centered a distance $S = 50$ Å from the center of the bilayer. The squared Fourier transforms B^2 and P^2, for the bilayer alone and the protein layer alone respectively, are shown by the dashed curves in Fig. 1 *b*; the solid curve is the squared Fourier transform of the compound profile.

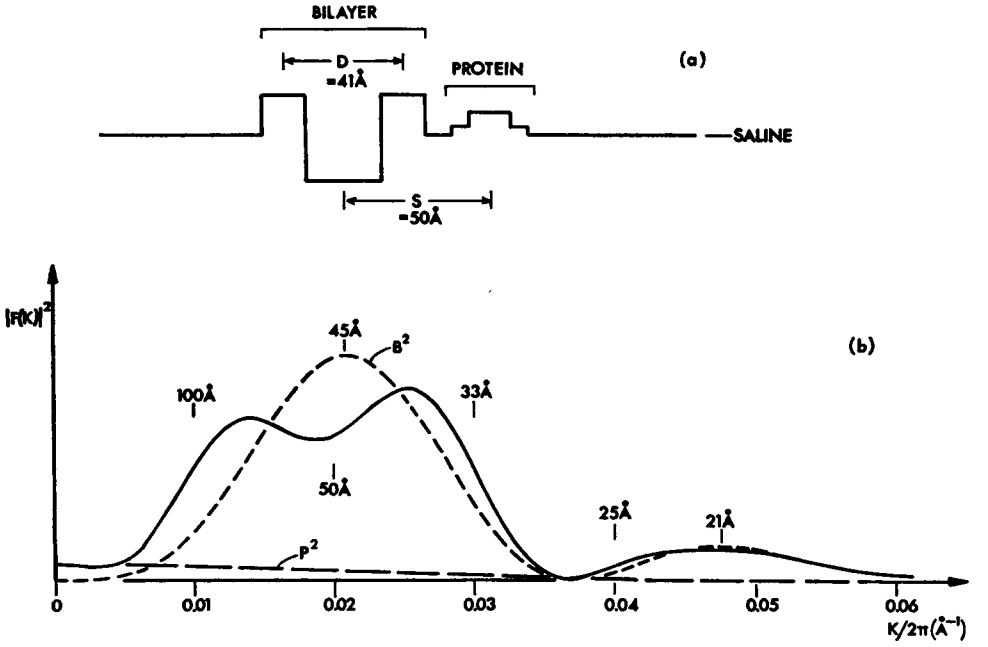

FIGURE 1 (*a*) Simplified electron density profiles for a bilayer and for a layer of 30 Å-diameter protein molecules to one side. The center-to-center distance is $S = 50$ Å. (*b*) The corresponding squared Fourier transforms: B^2 for the bilayer alone, P^2 for the protein layer alone, and the solid curve for the compound structure. The Bragg's law spacings for the successive maxima and minima of the cosine factor in expression 3 (100, 50, 33, 25, and 20 Å) locate oscillations about the B^2 curve.

Taking into account that B is negative over the bilayer band centered at 45 Å (see Wilkins et al., 1971) and that P is positive there, the cross-interference effects predicted by expression 3 are confirmed by comparing the solid and B^2 curves in Fig. 1 *b*. Over the second bilayer band, centered at 21 Å, B and P are both positive and the ripples are seen as predicted until they finally die out as P goes to zero.

Fig. 2 *a* shows the same bilayer profile as in Fig. 1 *a* but now with a layer of 60 Å-diameter protein molecules a distance $S = 145$ Å from the bilayer. Comparing Figs. 1 *b* and 2 *b*, two effects are apparent. First, one sees the higher-frequency ripple in Fig. 2 *b* due to the larger value of S. Also, in Fig. 2 *b* the ripple dies out nearer the origin because the protein layer is thicker. For both figures it is evident that, if the value of S had been unknown, it could have been estimated.

As indicated in the Discussion, there may in practice be broad intervals where

$$B(K)^2 \gg P(K)^2, \tag{4}$$

i.e., where the bilayer diffraction will be large compared with the protein diffraction. Figs. 1, 2, and 3 illustrate this case, save near the origin. Away from the origin, the principal effect of the protein is to cause the diffracted intensity to oscillate above and below the bilayer pattern in accord with expression 3.

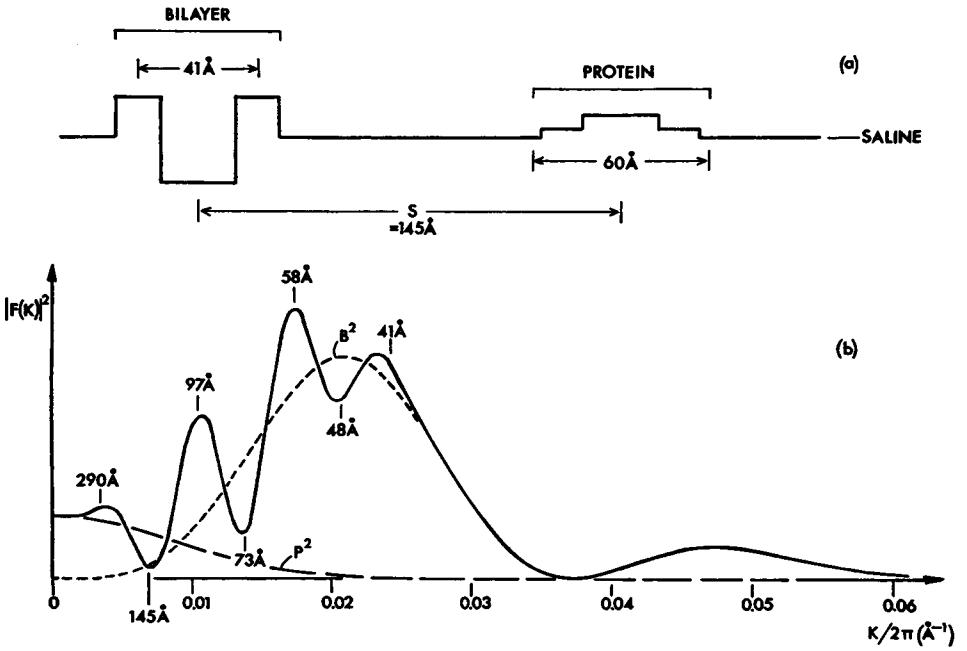

FIGURE 2 (*a*) The same bilayer profile as in Fig. 1 *a* but now with a simplified profile for a layer of 60 Å-diameter protein molecules farther away. (*b*) Corresponding squared Fourier transforms. The ripples in the solid curve are more closely spaced than in Fig. 1, since S is larger, and they die out at a larger Bragg's law spacing because the protein layer is thicker.

A striking consequence of inequality 4 is that there are distinct arrangements of the protein for which the diffraction patterns will be closely similar (Fig. 3). Thus, wherever the inequality is valid the principal contribution to expression 2 from the protein will be cross-interference terms of the form of expression 3. The sum of these terms will be the same whether a given amount of protein is all on one side of the bilayer (at $+S$) or distributed on both sides (at $\pm S$) in any proportions. In practice there will be an ambiguity in locating the protein from an observed cross-interference ripple unless diffraction data are recorded in a region where inequality 4 does not hold. Thus the two squared Fourier transforms in Fig. 3 *b* (solid and dashed lines) are decisively distinguished only near 240 Å, where Fig. 1 *b* shows that $B^2 < P^2$ (see also Blaurock, 1972 *c*).

Natural membranes have large amounts of protein, which can contribute more strongly than in Figs. 1, 2, and 3. Fig. 4 illustrates the case of a possible structure having a normal, continuous lipid bilayer and all the protein outside; the earlier Danielli-Davson (1935) model is of this kind. The simplified lipid bilayer profile in Fig. 4 *a* was calculated using the complete analysis given by Anderson and Maude

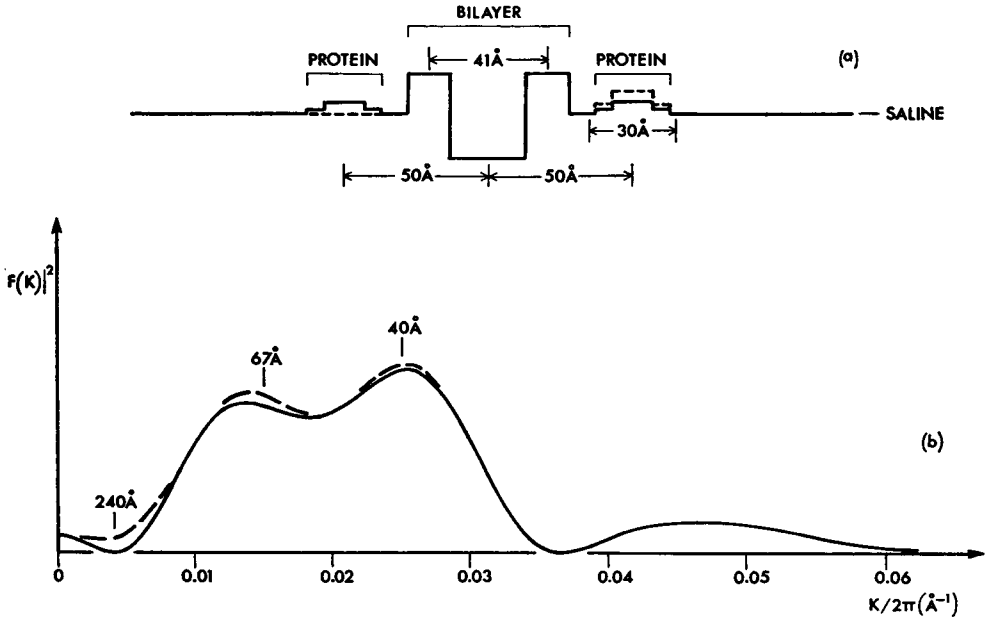

FIGURE 3 (*a*) The same bilayer profile as in Fig. 1 *a* but here the protein is distributed half on each side of the bilayer ($S = \pm 50$ Å). The profile of Fig. 1 *a* is indicated by the dashed line for comparison. (*b*) The corresponding squared Fourier transforms. The dashed curve here is the same as the solid curve in Fig. 1 *b*. Here there is a zero in the solid curve at about 240 Å, where the Fourier transform changes sign. In contrast, the dashed curve shows a nonzero minimum. This difference would clearly distinguish the symmetric from the asymmetric arrangement whereas the comparably small differences around 67 and 40 Å would be more difficult to analyze.

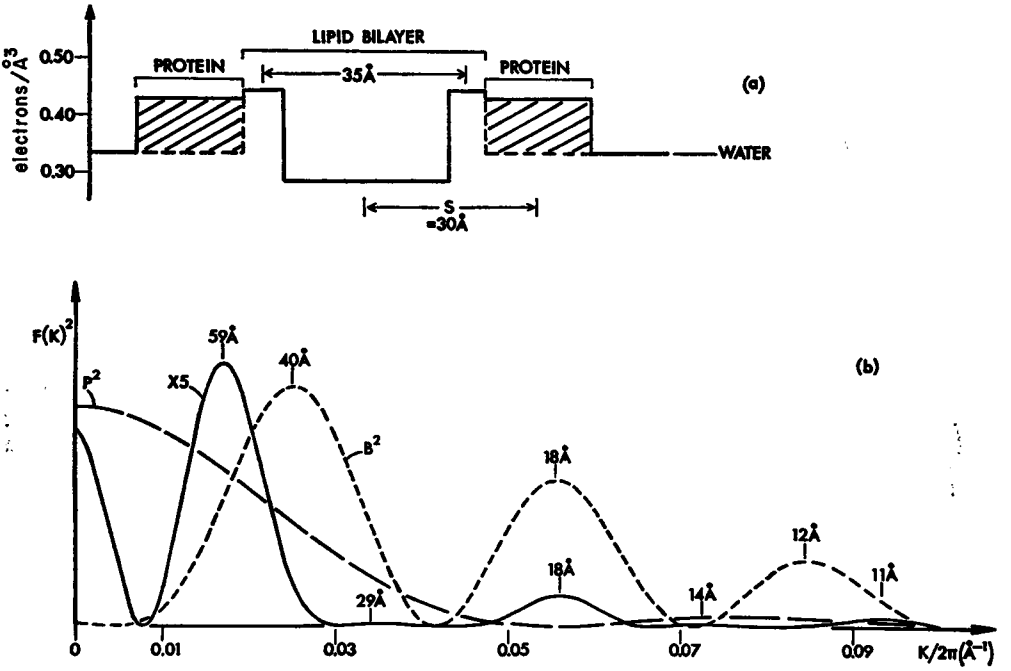

FIGURE 4 (*a*) Simplified profiles for a lipid bilayer with hydrated head group layers and for an equal volume of protein in two unhydrated, 18 Å-thick layers (see text for details). (*b*) The corresponding squared Fourier transforms; P^2 is for either layer of protein. For convenience, the solid curve is scaled down by a factor of 5.

(1970) for the phospholipids extracted from visual cell outer segments. The average area per molecule is assumed to be 70 Å² (see Luzzati et al., 1966), and the average head group and hydrocarbon chain volumes were calculated by the method of Traube (1899). The total volume of protein is assumed to be equal to that of the lipids; this is approximately the ratio for visual cell outer segments (see Blaurock and Wilkins, 1969). The layers of protein are assumed to be unhydrated, making them nearly as electron dense as the layers of hydrated lipid head groups.

In Fig. 4 *b* the effect of the added protein is simply to increase the bilayer *D* value; the Bragg's law spacings given at the centers of the bands (59, 29, 18, 14, and 11 Å) indicate a *D* value of 56 Å. The sum of the two peaks at 60 Å predicted by expression 3 for cross interference between the bilayer and each layer of protein coincides with a peak, half as high, for cross interference between the two protein layers. Thus a strong cross-interference effect between lipid and protein is present, but there is not an obvious ripple. As indicated in the Appendix, the model is not valid for the disk membrane and some other natural membranes because the apparent *D* value, 56 Å, is too large.

In the more general case the profiles of both the bilayer and the protein layer will be asymmetric. In this case expression 2 is replaced by:

$$| B |^2 + | P |^2 + 2 \cdot | B | \cdot | P | \cdot \cos (K \cdot S + \Phi_B - \Phi_P). \quad (5)$$

In this case S may be defined as the distance to the center of mass of the protein. Φ_B is the phase angle defined by the equation

$$B(K) = | B(K) | \cdot \exp (i \cdot \Phi_B[K]); \quad (6)$$

a similar equation holds true for $\Phi_P(K)$. Expression 5 is almost the same as expression 2, the difference being that the term $(\Phi_B - \Phi_P)$ will shift the peaks and troughs of the cross-interference term from the positions for symmetric profiles, when Φ_B and Φ_P are equal to 0 or π. Large departure from symmetry of either the bilayer or the protein-layer profile will result in large shifts.

DISCUSSION

The amino acid residues in a protein are all considerably more electron dense than water. (Average densities of 0.37–0.51 electrons/Å^3 [Blaurock, 1972 *a*] are calculated using the residue volumes [including the peptide backbone] of Cohn and Edsall [1943]; the value for water is 0.334 electrons/Å^3.) Consequently, the principal contrast leading to X-ray diffraction at low angles is between the average electron densities of the protein and of the surrounding water. Therefore the diffracted intensity will be strongest near the origin and will drop to a low value near the Bragg's law spacing equal to the half-width of the protein-layer profile; beyond, there may be small subsidiary bands.

For a lipid bilayer, the principal contrast is between the average electron densities of the head groups and of the fatty chains. (Values near 0.50 electrons/Å^3 are calculated for unhydrated phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine head groups using Traube's [1899] volumes; the value for the liquid paraffin-like state of the fatty chains is somewhat less than 0.30 electrons/Å^3.) Closest to the origin the diffracted intensity may be small, but for well defined layers of head groups there will be an extensive series of diffraction bands (Wilkins et al., 1971). Thus inequality 4 is least likely to be met near the origin and near the nodes separating the bilayer bands but is likely to be met over much of each bilayer band. Also, the bilayer will tend to dominate because the lipid molecules will be closely packed whereas the density of protein molecules outside the bilayer can be small.

The particular bilayer profiles in Figs. 1–4 are not thought to be valid for any lipid bilayer. Nonetheless the model profiles show the essential features of the bilayer: two electron-dense layers separated by a region of low density (Wilkins et al., 1971). Varying the precise form of the bilayer peaks or of the trough between will not cause the bilayer diffraction bands (see Introduction) to change their general form although the relative amplitudes of the bands will change. The protein-layer profiles in Figs. 1–3 are a convenient and broadly faithful approximation to the parabolic profile for a uniformly dense, ellipsoidal protein molecule.

For a specimen to test the theory, lipid vesicles were incubated with cytochrome *c*. The results given in the following paper (Blaurock, 1973) prove the validity of the theory developed here.

The specimen for which the theory was principally developed is the envelope of *H. halobium*, consisting of a plasma membrane and a wall of protein (Stoeckenius and Rowen, 1967). By comparing the envelope pattern and the simpler bilayer pattern recorded from the wall-free membrane, a low-resolution profile was derived consisting of the bilayer and both layers of protein shown in Figs. 1 *a* and 2 *a* (A. E. Blaurock, D. Oesterhelt, and W. Stoeckenius, manuscript in preparation).

Since the scattering properties of the protein are likely to be known, the layer of protein molecules can be used in the way a heavy atom is used in protein crystallography (see, for example, Dickerson, 1964). I note that the theory of Lesslauer and Blasie (1971), developed for a specimen having two kinds of bilayer, also uses a cross-interference effect to determine phase angles.

This analysis of a rather special structure may be useful in attempts to locate the protein in natural membranes. The problem is discussed further in the Appendix.

APPENDIX

Consideration of Some Natural Membranes

The low-angle diffraction patterns recorded from erythrocyte ghosts, nerve-ending vesicles, *Mycoplasma* membrane, and the purple and red membrane fractions from *H. halobium* are of the bilayer form with D values generally in the range 40–45 Å (Wilkins et al., 1971; Engelman, 1971; Blaurock and Stoeckenius, 1971). The spacings and relative intensities of the bands indicate that these membranes have a bilayer profile, and the two electron-dense layers probably locate lipid head groups (Wilkins et al., 1971).

For a membrane that is half protein, the presence of an uninterrupted lipid bilayer with D = 40–45 Å would require that the protein form substantial layers (upwards of 20 Å thick) on one or both surfaces of the bilayer. With all the membrane protein in fixed positions, the protein profile will be a prominent, if asymmetric, peak with a definite value for S; this is so whether or not the individual molecules cover a broad range of diameters and distances from the bilayer. Thus there will be a large cross-interference effect (for an example see Fig. 1 in Engelman, 1971).

Fig. 4 illustrates one possibility. However, the value D = 56 Å derived from the solid curve in Fig. 4 *b* is considerably larger than the value D = 40 Å found for the disk membrane in intact visual cells (Blaurock and Wilkins, 1969, 1972). Therefore, this structure does not account for the disk membrane pattern. For the other natural membranes this structure will be similarly unsatisfactory.

If, alternatively, appreciable amounts of water were present in the protein layers, then S would increase, from 0.86 times the peak-to-peak distance for the lipid bilayer in Fig. 4 *a* to a value equal to or greater than this distance. According to expression 3 or its equivalent in expression 5, the frequency of the ripple would be at least twice that of the bilayer pattern itself. However, for the natural membranes listed the predicted cross-interference effects are not evident. Thus, because large changes from the pattern for the lipids alone are predicted, neither form of the model having a normal and continuous bilayer accounts for the patterns

recorded from several natural membranes. The possibility that the predicted ripple is lost due to relative motion of the bilayer and the protein layer seems unlikely.

As an alternative, a structure having protein molecules half-submerged in the lipid bilayer can be considered. This form has been proposed for the disk membrane (Wilkins et al., 1971; Blaurock, 1972 *a*, *b*; Vanderkooi and Sundaralingham, 1970) and for the purple membrane (Blaurock, 1972 *b*), where there is an unusually high electron density at the center of the bilayer profile (Blaurock and Stoeckenius, 1971). Expression 2 can be applied by identifying P with the Fourier transform of the contrast between protein and the hydrated bilayer. In this case S will be equal to $D/2$, and the cross-interference term will not be evident since it will have the same frequency as the bilayer bands. The model implies an apparent thinning of the lipid bilayer, in agreement with measured values (Wilkins et al., 1971; Blaurock and Stoeckenius, 1971; Blaurock, 1972 *c*).

I thank Doctors D. L. D. Caspar and D. M. Engelman for stimulating discussion and Doctors W. Stoeckenius, V. K. Miyamoto, and M. H. F. Wilkins for helpful criticism of the manuscript.

This work was supported by Program Project Grant HE 06285 from the National Heart and Lung Institute.

Received for publication 30 July 1972 and in revised form 19 October 1972.

REFERENCES

ANDERSON, R. E., and M. B. MAUDE. 1970. Biochemistry. **9**:3624.
BLAUROCK, A. E. 1972 *a*. *Adv. Exp. Med. Biol.* **24**:53.
BLAUROCK, A. E. 1972 *b*. *Chem. Phys. Lipids.* **8**:285.
BLAUROCK, A. E. 1972 *c*. *Nature* (*Lond.*). **240**:556.
BLAUROCK, A. E. 1973. *Biophys. J.* **13**:290.
BLAUROCK, A. E., and W. STOECKENIUS. 1971. *Nature* (*Lond.*). **233**:152.
BLAUROCK, A. E., and M. H. F. WILKINS. 1969. *Nature* (*Lond*). **223**:906.
BLAUROCK, A. E., and M. H. F. WILKINS. 1972. *Nature* (*Lond*). **236**:313.
COHN, E. J., and J. T. EDSALL, editors. 1943. Proteins, Amino Acids and Polypeptides. Reinhold Publishing Corp., New York.
DANIELLI, J. F., and H. DAVSON. 1935. *J. Cell. Comp. Physiol.* **5**:495.
DICKERSON, R. E. 1964. The Proteins. H. Neurath, editor. Academic Press Inc., New York. 2nd edition. **2**:603.
ENGELMAN, D. M. 1971. *J. Mol. Biol.* **58**:153.
LESSLAUER, W., and J. K. BLASIE. 1971. *Acta Crystallogr. Sect. A.* **27**:456.
LEVINE, Y. K., and M. H. F. WILKINS. 1971. *Nat. New Biol.* **230**:69.
LUZZATI, V., F. REISS-HUSSON, E. RIVAS, and T. GULIK-KRZYWICKI. 1966. *Ann. N.Y. Acad. Sci.* **137**:409.
RAND, R. P., and V. LUZZATI. 1968. *Biophys. J.* **8**:125.
STOECKENIUS, W., and R. ROWEN. 1967. *J. Cell Biol.* **34**:365.
TRAUBE, J. 1899. *Samml. Chem. Chem.-Tech. Vortr.* **4**:255.
VANDERKOOI, G., and M. SUNDARALINGAM. 1970. *Proc. Natl. Acad. Sci. U.S.A.* **67**:233.
WILKINS, M. H. F., A. E. BLAUROCK, and D. M. ENGELMAN. 1971. *Nat. New Biol.* **230**:72.