THE STRUCTURE OF A LIPID-CYTOCHROME *c* MEMBRANE

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ABSTRACT In the preceding paper a theory has been developed for the X-ray diffraction pattern from a lipid bilayer coated with protein molecules. In this paper the validity of the theory is tested. After adding cytochrome *c* to a preparation of lipid vesicles both vesicles and, in lesser amount, a multilayered structure are found. The expected cross-interference ripple is evident, and a computed profile shows the added protein at the surface of an unchanged lipid bilayer. The results confirm the structure proposed by others on the basis of rather indirect chemical and X-ray data. Protein coating a lipid bilayer is a possible structure for natural membranes. These results demonstrate that globular protein molecules can be detected at the surfaces of a lipid bilayer.

INTRODUCTION

In the preceding paper (Blaurock, 1973) the X-ray diffraction pattern has been predicted for a lipid bilayer with a layer of protein molecules added outside. For smaller amounts of protein, a ripple will be introduced into the simple bilayer pattern; the amplitude of the ripple is proportional to the amount of protein and its frequency is proportional to the distance from the bilayer to the protein layer.

On the basis of lipid and protein thicknesses calculated for multilayer specimens, and other evidence, Gulik-Krzywicki et al. (1969) have proposed that cytochrome *c* may be located in layers at the surfaces of a phospholipid bilayer. A similar specimen was prepared in order to apply the preceding theory (Blaurock, 1973) to test the structure they propose.

After adding cytochrome *c* to lipid vesicles, a ripple is observed as predicted for cross interference between the bilayer and a layer of protein molecules. Thus the structure proposed by Gulik-Krzywicki et al. (1969) and Shipley et al. (1969) is confirmed, and for the first time the protein molecules are seen at the surfaces of the bilayer (see also Blaurock, 1972).

MATERIALS AND METHODS

The lipid-cytochrome *c* specimen was kindly prepared by Dr. V. K. Miyamoto, as follows. Lipids (including phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and

phosphatidic acid) were precipitated by adding acetone to Asolectin (3.1% P by weight) dissolved in chloroform. The precipitated lipids were then sonicated in 0.25 M sucrose, buffered at pH 7.1 with Tris, to make single-walled vesicles (Miyamoto and Stoeckenius, 1971). Horse heart cytochrome *c* (Sigma Type VI, Sigma Chemical Co., St. Louis, Mo.) was dialyzed against the buffer. Then the vesicles were incubated overnight at 4°C with cytochrome *c* in the proportion 1 mol of protein to 64 mol of lipid phosphorus (protein:lipid weight ratio of 0.2:1). The mixture was then passed through a column of Sephadex G-100. The lipid-protein mixture appeared in its original proportions in the void volume, and no free protein peak was found. The complex was concentrated by centrifuging at 400,000 *g* for 1 h. A portion of the same vesicles, but without cytochrome *c*, was also centrifuged.

To make an X-ray specimen, material from a centrifuge pellet was drawn into a 1 mm glass capillary ($\frac{1}{100}$ mm wall; Unimex-Caine, Griffith, Ind.), which was then flame sealed to be airtight. A point-focusing, low-angle X-ray camera of the Franks (1955) type was used. To have the convenience of an optical bench, camera components were mounted on two parallel rails and the whole was enclosed by cylindrical covers as in the Elliott (1965) toroid camera. The Cu K_α radiation used was generated in a Jarrel Ash microfocus unit (Jarrel Ash Div., Fisher Scientific Co., Waltham, Mass.). The low-power, line-focus filament was horizontal, and its image was viewed at an angle of about 5° from the target surface to provide an X-ray source about 0.1 mm in diameter. The window of the tube was Ni foil, ensuring that K_β radiation was negligible.

Exposures were made under vacuum to eliminate air scatter. Parasitic scatter was found to be negligible, and all recorded intensity was assumed to come from the specimen. Exposures were at room temperature (~25°C). The specimen-to-film distance was either 8 cm, for best resolution towards the center of the pattern, or else 4 cm; when recording diffuse diffraction, the shorter distance increased exposure speed by a factor of 4. Film density was recorded using a Joyce, Loebl microdensitometer (Mark III C, Joyce, Loebl & Co., Inc., Burlington, Mass.). Areas of reflections on a densitometer tracing were measured with a planimeter.

RESULTS

Before the cytochrome *c* was added, the sonicated lipids gave a pattern of the form predicted by Wilkins et al. (1971) for lipid bilayers; the continuous curve in Fig. 1 shows a densitometer tracing of the first three bands plotted after correcting for disorientation. The Bragg's law spacings[1] given for the centers of the bands indicate that the two electron-dense head group layers are somewhat under 40 Å apart. A profile was computed for data from the first band alone (the solid curve in Fig. 3).

After cytochrome *c* was added, a series of sharp rings was recorded, superimposed on a diffuse background, Fig. 2. The sharp rings are Bragg's law orders of an 84 Å periodicity and indicate that regions with a multilayered structure had formed. The diffuse scatter, which predominates in Fig. 2, indicates that there was still mainly unstacked material, probably vesicles with small amounts of cytochrome *c* adsorbed (see Discussion). As described in the legend to Fig. 2, two sets of diffracted intensities were measured on the tracing: (1) for the multilayers alone (●) and (2) for the total material, i.e., both multilayers and unstacked membranes together (○). Both sets of corrected, integrated intensities are plotted in Fig. 1.

[1] See the footnote to the Introduction in the preceding paper (Blaurock, 1973).

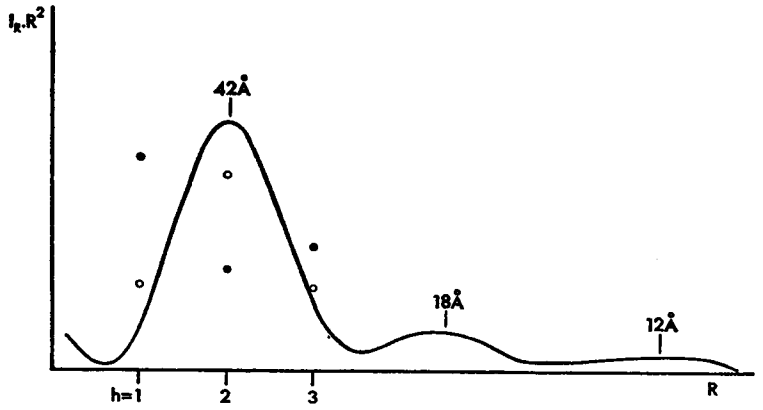

FIGURE 1 The continuous curve (——) plotted for the lipid vesicles, after correcting (Wilkins et al., 1971) a radial densitometer trace, I_R , by a factor of R^2. In a low-angle pattern R, the distance from the center of the diffraction pattern, is directly proportional to K. Ordinates are in relative units. The first, second, and third bilayer bands are centered at Bragg's law spacings of 42, 18, and 12 Å respectively. The circles (● and ○) are the two sets of integrated intensities (see Fig. 2) for the lipid-cytochrome *c* membranes plotted after correcting by h^2 and scaling as in Fig. 3. h is the order number for the 84 Å period.

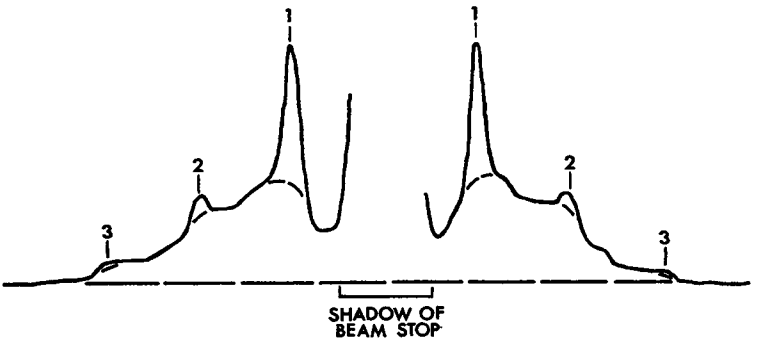

FIGURE 2 Radial densitometer trace of the low-angle diffraction pattern after adding cytochrome *c* to the lipid vesicles. The first three orders of a Bragg periodicity of 84 Å are superimposed on a continuous background. Two sets of areas were measured: (1) of the sharp, multilayer peaks alone (base lines shown by short dashes), giving the closed circles (●) in Fig. 1; and (2) of the total diffraction by including the diffuse, nonmultilayer diffraction with the nearest sharp reflection (base line shown by long dashes), giving the open circles (○) in Fig. 1. The validity of the second way is considered in the Discussion. The specimen-to-film distance was 80 mm.

For comparison, note that Gulik-Krzywicki et al. (1969) mixed phosphatidylinositol, cytochrome *c*, and water and found a 79 Å periodicity. The 5 Å difference from my observation is due principally to the different lipid mixture used, for an 87 Å periodicity was found when they substituted mitochondrial lipids. Shipley et al. (1969) found an 87 Å periodicity for cytochrome *c* and yet another lipid mixture, and their Fig. 1 *b* shows the intensities of the Bragg reflections decreasing with

increasing order number, in agreement with Fig. 2 in this paper. I have not observed the larger periodicity that they sometimes recorded (116 Å) and that Gulik-Krzywicki et al. also found (112 Å).

It was intended to record purely continuous diffraction from lipid vesicles with adsorbed cytochrome *c*. However, the multilayer structure formed spontaneously from part of the lipids and protein. The resulting Bragg reflections will have sampled the continuous, squared Fourier transform for the profile of a single lipid-protein membrane. Consequently comparison with the diffracted intensity from the lipid vesicles alone is still possible. Moreover the observed period helps to define the structure, as follows.

Dimensions of 25 by 25 by 37 Å were determined by Dickerson et al. (1967) for the spheroidal horse heart cytochrome *c* molecule in a crystal. Assuming one layer of cytochrome *c* molecules in the 84 Å period, there remains 47–59 Å for a lipid bilayer. The bilayer in the lipid vesicles clearly is thicker than the 36 Å peak-to-peak distance seen in Fig. 3. An estimate for the thickness is the periodicity of egg lecithin multilayers: 51–64 Å, depending on the water content (Small, 1967). Thus, the 47–59 Å space is sufficient for a normal lipid bilayer.

An alternative model would have two layers of cytochrome *c* molecules between adjacent bilayers; each bilayer would then be no more than 34 Å thick. However, this alternative does not satisfy the finding by Gulik-Krzywicki et al. (1969) of a normal lipid "partial thickness" of 40–43 Å excluding the substantial amounts of water and protein. Therefore only the structure having normal lipid bilayers alternating with single layers of cytochrome *c* molecules will be considered further. A ripple for cross interference between bilayer and protein layer is predicted as follows.

The diffraction from the proposed structure will be proportional to the squared Fourier transform of the compound profile (see expressions 2 and 5 in the preceding paper). The profiles for the bilayer and protein layer in the proposed structure will be precisely symmetric or else nearly so (see Discussion). Ignoring possible asymmetric detail in the profiles and taking S equal to half the X-ray period, expression 2 becomes:

$$B^2(K) + P^2(K) + 2 \cdot B(K) \cdot P(K) \cdot \cos (K \cdot 42 \text{ Å}). \qquad (1)$$

The first two terms are for the bilayer and protein layer, respectively, and the third term is for cross interference.

First, to agree with previous results (Wilkins et al., 1971), $B(K)$ is assumed to be negative (phase angle π) over the first lipid-vesicle band in Fig. 1. Secondly, protein is generally more electron dense than water (see Discussion in the preceding paper). Accordingly $P(K)$ for the cytochrome *c* spheroid will be positive (phase angle 0) at the origin and will decrease monotonically to zero at a Bragg's law spacing of about 25 Å or less, depending on orientation (see p. 359 of Guinier, 1963, for the case of a uniform sphere). Thirdly, for a given order h of the 84 Å period the cosine factor in

expression 1 is $(-1)^h$. Thus the cross-interference term will increase the intensity at order 1 and decrease it at order 2, both substantially; any change at order 3 will be small. Fourth, the term $P^2(K)$ will decrease monotonically over the first three orders. Together, the second and third terms in expression 1 will give a large increase in intensity at order 1. At order 2 there will be a decrease or small increase accordingly as $P(K)$ is less than $2 \cdot B(K)$ or greater. In either case, the ratio of the intensities at orders 1 and 2 will increase. The reader is referred to Fig. 1 in the preceding paper to see the ripple for a similar structure.

The comparison of ratios in Table I confirms the predicted effects. Also, a ripple is evident in Fig. 1, as predicted for cross interference. Thus the observed diffraction confirms prediction for a normal lipid bilayer with protein outside.

The profile of the proposed lipid-protein membrane in the multilayer structure can equally be considered as the symmetric arrangement of the bilayer profile with protein peaks half as high on each side. Then, as can be demonstrated by gradually increasing the protein content from zero, the phase angles needed to compute the corresponding symmetric Fourier synthesis are likely to be those for the bilayer alone, i.e. π for $h = 1$, 2, and 3. Using these phase angles and the square roots of the values plotted in Fig. 1 for $h = 1$, 2, and 3, Fourier syntheses were computed for the lipid vesicles and for the lipid-cytochrome *c* membrane (Fig. 3).

In Fig. 3, half of the symmetric Fourier synthesis computed for the lipid-cytochrome *c* multilayer data is shown to the right of center (●). The synthesis includes a bilayer profile which superimposes well on the bilayer profile for the lipid vesicles (plain curve). As indicated in the Discussion, none of the other seven possible symmetric Fourier syntheses includes the same bilayer profile as for the vesicles alone. Outside the bilayer the two curves separate, and the cytochrome *c* molecules are recognized by the increased electron density in comparison with the indicated water level. The difference profile in Fig. 3 *b* (●) may be compared to an inverted, truncated parabola, 30 Å across the base, computed for uniform, unoriented protein molecules of weight 12,400 g/mol. Results for the data including the diffuse, nonmultilayer diffraction (○) are much the same except that the peak identified in Fig. 3 with protein is smaller.

TABLE I

RATIOS OF THE CORRECTED INTENSITIES OF ORDERS 1, 2, AND 3 OF THE 84 Å PERIODICITY

	F_1^2/F_2^2	F_2^2/F_3^2
Lipid vesicles*	0.17	3.5
Bragg reflections and diffuse background (○ in Fig. 1)	0.4	2.3
Bragg reflections alone (● in Fig. 1)	2.1	0.8

* For values taken from the corresponding three points on the continuous curve in Fig. 1.

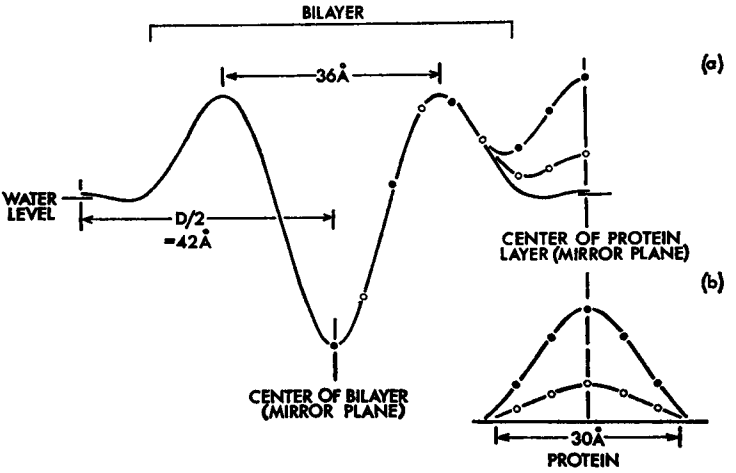

FIGURE 3 (*a*) Symmetric Fourier syntheses showing electron density profiles for the lipid vesicle wall (plain curve) and, to the right of center, halves of two profiles for the lipid-cytochrome *c* membrane (● and ○; see Fig. 2). Magnitudes for the 84 Å periodicity are the square roots of values plotted in Fig. 1, and the phase angles are π, π, π in all cases. The ordinates (in arbitrary units) have been scaled to make the bilayer profiles coincide. The resolution is 14 Å. (*b*) (Lower right.) Differences between the upper two profiles and the lower one in *a*. The 30 Å average diameter computed for unoriented cytochrome *c* molecules is indicated. The protein layer to the left of the bilayer is omitted for clarity.

DISCUSSION

The structure proposed for the lipid-cytochrome *c* membrane fits into the 84 Å-thick space observed for the multilayers. The regular changes in diffracted intensity predicted for a layer of protein added at the surface of a normal lipid bilayer are confirmed in Table I, and the predicted ripple is evident in Fig. 1 (cf. Fig. 1 in the preceding paper). Fig. 3 shows that the vesicle-wall profile is not changed by the added cytochrome *c*, in agreement with the finding by Gulik-Krzywicki et al. (1969) that the partial thickness of lipid in multilayers did not change after cytochrome *c* was added. These results confirm the general correctness of the structure proposed by them and pictured for the first time in Fig. 3.

As noted in Results, the other seven possible symmetric profiles for the lipid-cytochrome *c* membrane have also been examined. Fig. 4 shows a synthesis for the multilayer data using an alternate set of phase angles. Using Figs. 3 and 4, the reader can examine all eight possible profiles by alternately identifying the origin or the half-period point with the center of the membrane, before or after inverting the curves. Only for the identification made in Fig. 3 is there a largely unchanged bilayer profile.

In principle both the bilayer and the protein layer could be asymmetric in the proposed structure, and asymmetric electron density profiles have not been ruled out. However, Dickerson et al. (1967) report an arrangement of the amino acid

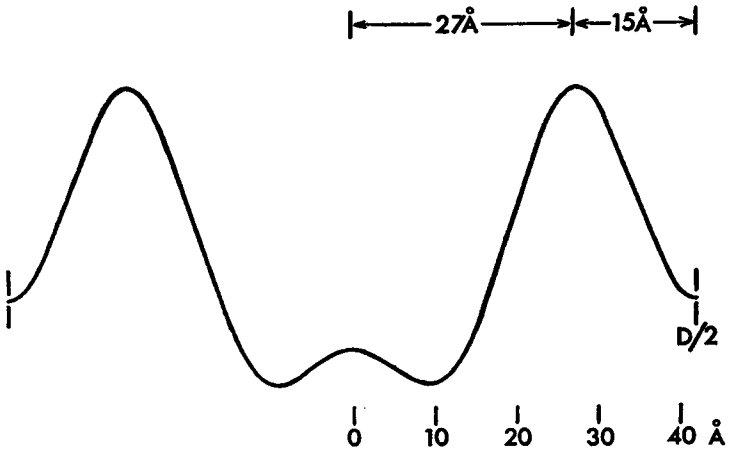

FIGURE 4 Fourier synthesis computed as for the closed circles (●) in Fig. 3 but with an alternate set of phase angles (π, π, 0). As in Fig. 3 there are mirror planes at 0 and $D/2$. As indicated in the Discussion, the syntheses in Figs. 3 and 4 can be used to see all eight possible symmetric profiles. The identification in Fig. 3 is the only one giving an unchanged bilayer profile.

residues in cytochrome *c* giving a comparatively symmetric profile for any orientation of the spheroid: the lower density, hence more hydrophobic, residues are located in the core of the molecule with the higher-density, hydrophilic residues at the surface. In much the same way, the lipid bilayer profile would be largely symmetric even if there were chemical asymmetry (see Discussion in the preceding paper). Thus the derived profile is believed to be accurate except, possibly, in details of the protein and lipid-head group profiles.

The cross-interference term in expression 1 is directly proportional to the amount of protein present and therefore will be the same whether the protein is distributed uniformly on all the vesicles or else is concentrated on some vesicles and absent from others. With this in mind, in the one case (○ in Figs. 1 and 3) I have included the diffuse scatter with the measured intensities of the sharp multilayer reflections. The larger protein-to-lipid ratio found in the multilayers than in the specimen as a whole (Fig. 3) suggests that the diffuse scatter comes from vesicles with too little cytochrome *c* for them to have combined into multilayers. These vesicles probably have cytochrome *c* molecules only on the outside surfaces. However the possibility that the protein molecules have reached the inside surfaces as well, although unlikely, has not been ruled out; the present data are not adequate to determine the point.

Considering the multilayer profile in Fig. 3 *a* (●) and taking the water level as a base line, the areas under the protein and lipid-head group peaks measure the amounts of lipid and protein present. Based on volumes and numbers of electrons calculated for the head groups of phosphatidylethanolamine and phosphatidylcholine and for some proteins (unpublished calculations; see Cohn and Edsall, 1943,

and Traube, 1899), a rough proportion of 0.5:1 is estimated for the weight ratio of protein to lipid. A ratio of 0.78:1 was determined chemically by Gulik-Krzywicki et al. (1969) for a different lipid. Including the diffuse, nonmultilayer scatter (○ in Fig. 3) the estimated weight ratio is roughly 0.2:1. The orientation of the 37 Å-long diameter of the spheroidal cytochrome *c* molecule is not evident at the present resolution. The molecules may be unoriented.

An alternative structure for the multilayer, having two hydrated lipid-protein membranes in the 84 Å-thick space, has also been considered. The collapse of a vesicle with an asymmetric wall could, for example, give rise to a pair of membranes. That there is sufficient lipid for only one normal bilayer (Gulik-Krzywicki et al., 1969) rules out the presence of two normal bilayers. Nonetheless the possibility remains of a pair of membranes in each of which the lipid partial thickness would be about 20 Å; the two membranes together would account for the 40–43 Å value found by Gulik-Krzywicki et al. (1969). The proposed structure, Fig. 3, accounts for the separation of the lipid from the cytochrome *c* in saline (Gulik-Krzywicki et al. 1969) as well as for the diffraction data (Fig. 1). In contrast, the alternative structure seems unlikely, but it has not been ruled out.

Assuming the correctness of the structure derived for the multilayers, Fig. 3, it follows that an appreciable fraction of the vesicles has collapsed and possibly ruptured after cytochrome *c* was added and also that the protein molecules have reached both sides of these lipid membranes. Clearly, any explanation of the process must take into account that the bilayer structure is changed little, if at all, by the added protein. Nonetheless the force of attraction between negatively charged lipid molecules and the positively charged protein molecules (isoelectric point at pH 10–11; see Spector, 1956, p. 24) probably causes the vesicles to clump together after the protein is adsorbed and then may provide the energy for them to rupture and form the multilayers.

In summary, the theory in the preceding paper accounts for the effect on the observed bilayer diffraction pattern due to added protein molecules, and layers of them have been seen on the surfaces of the lipid bilayer. This method is available to look for similar structure in natural membranes.

I thank Dr. V. K. Miyamoto for generously preparing specimens, Dr. W. Stoeckenius for laboratory facilities and encouragement, and Dr. W. R. Lieb and Dr. 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.

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