LIQUID DIFFRACTION ANALYSIS OF SARCOPLASMIC RETICULUM

II. Solvent Electron Contrast Variation

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ABSTRACT Intensities of x-ray scattering from rabbit muscle sarcoplasmic reticulum membrane have been measured over the range of $s = 0.05$ – 0.25 , at solvent densities varying between $\rho_0 = 0.335$ and 0.389 electrons/ $\text{\AA}^3$. Analysis of the results shows agreement with the elements of structure deduced earlier by a different technique, based on the variation of the lipid-protein concentration ratio. In addition, the present work extends the analysis to allow isolation of the lipid contribution to the total scattering, from which a profile of the lipid electron density normal to the membrane face is evaluated. The scattering arising from electron correlations within the plane of the bilayer has also been identified.

INTRODUCTION

Sarcoplasmic reticulum (SR) of skeletal muscle is a good model for analyzing the structure of a biological membrane because of its relatively simple function and composition (Tada et al., 1978). Phospholipids make up >95% of the lipid, with phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol accounting for ~70%, 15%, and 10%, respectively. One protein, the Ca^{++} -stimulated, Mg^{++} -dependent ATPase (Ca^{++} -ATPase) accounts for ~90% of the total membrane protein (Meissner, 1975). This protein controls muscle relaxation through ATP-dependent uptake of calcium from the muscle myofibrillar space.

In the preceding paper of this series (Brady et al., 1981a), we presented an analysis of small-angle x-ray scattering from suspensions of rabbit muscle sarcoplasmic reticulum. By varying the relative concentrations of lipid and protein in the membranes, we identified the contributions of protein, lipid, and a protein-lipid interaction term to the total scattering. Analysis of the separated components indicated that the ATPase is an elongated particle (a cylinder or prolate ellipsoid) with a 4:1 axial ratio. The ATPase was also shown to be predominantly monomeric. Using the cylindrical shape for the protein molecule, we analyzed the protein-lipid scattering interaction term to show that the protein was inserted in the lipid bilayer in a highly asymmetric fashion, with most of its length protruding from one face of the bilayer. Since the lipid

mole fraction in the samples was low, and since the electron density of the buffer used as solvent approximately matched the average electron density of the lipid, both factors acting to produce a relatively weak contribution to the scattering (see below, Theory) from this component, we did not proceed further than identifying the lipid scattering with a (relatively) weak band centered at $s = 0.185 \text{ \AA}^{-1}$, which corresponds to a correlation distance of $41 \text{ \AA}$ in real space.

In this study we report a comparison of the scattering from a series of identical SR samples suspended in solvents that differ in electron density. The results are consistent with the data of our previous study with regard to protein structure and the protein-lipid scattering interaction. The contribution of lipid to the total scattering has been isolated, and used to derive a profile of the lipid electron density normal to the membrane face. The scattering arising from correlations within the plane of the bilayer has been identified. The use of the contrast-variation method, therefore, has allowed us both to confirm the earlier findings and to complete the analysis of the scattering from SR membranes initiated in the previous study.

MATERIALS AND METHODS

Preparation of Membranes

Intermediate density rabbit skeletal muscle SR vesicles prepared according to the method of Meissner (1975) were partially delipidated with the use of cholate as previously described (Meissner et al., 1973; Brady et al.,

1981a). Vesicles were suspended at 0°C at a protein concentration of 4.5 mg protein/ml in 0.3 M sucrose, 0.45 M KCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Tris, pH 8.0. Sodium cholate was added to the vesicle suspension at a final concentration of 5 mg/ml to remove membrane-associated protein and part of the phospholipid. The membranes were recovered by centrifugation for 2 h at 33,000 rpm in a Beckman 35 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Pellets were resuspended in a Potter-Elvehjem hand homogenizer in 0.3 M sucrose, 50 mM KCl, 1 mM HEPES, pH 7.4, dialyzed overnight at 4°C against the same buffer, and then placed on a linear 20–50% sucrose gradient containing 1 mM KCl and 1 mM HEPES, pH 7.4. After centrifugation for 200 min at 38,000 rpm in a Beckman SW41 rotor, membranes present in the 41–46% region of the gradient were collected, divided into five aliquots, and diluted with a 1 mM KCl, 1 mM HEPES, pH 7.4, buffer. Membranes were pelleted by centrifugation for 90 min at 33,000 rpm in a Beckman 35 rotor and resuspended in a 1 mM KCl, 1 mM HEPES, pH 7.4, buffer containing sucrose at a final concentration of sucrose of 0, 10, 20, 30, and 40.5% (wt/wt). The resulting membrane fraction was enriched with respect to the Ca²⁺-ATPase (90–95% of the protein). Its phospholipid composition was similar to that of the native membrane (Moore et al., 1978). The molar ratio of phospholipid to Ca²⁺-ATPase (mol wt 115,000) was estimated to be 44. Membranes (25–45 mg protein/ml) were stored at –65°C before use.

Data Collection and Analysis

The data were taken with a position-sensitive detector. A sample-to-detector distance of 50 cm was used for most of the measurements, but the data at the lowest angles were rechecked by increasing this distance to 100 cm. The main beam (CuK α radiation, $\lambda = 1.54 \text{ \AA}$) was collimated by two pinholes 0.75 mm in diameter, separated by 50 cm. 120 channels were used, covering the scattering range between $s = 0.03$ and 0.44 ($s = 4\pi/\lambda \sin \theta$, where λ is the wavelength and θ is one-half the scattering angle). The experimental temperature was 21°C. The data were collected in an 11/03 DEC microprocessor (DEC International, Inc., Madison, Wis.), and after the run was terminated they were transferred on command to a PDP 8/E minicomputer (Digital Equipment Corp., Maynard, Mass.), where they were averaged over a five-channel interval by a least-squares program. Each step of the procedure could be monitored by a printout or an oscilloscope display. The samples were contained in a cell fitted with 1-mil mica windows and had a thickness of 1 mm. Absorption corrections were negligible. No deterioration or settling of the sample was observed, as demonstrated by a constant counting rate at all scattering angles during the course of each experiment. The apparatus was monitored repeatedly by measuring the intensities and positions of the reflections from a lead stearate sample, and these varied by <2%. Since sucrose contributed to background scattering, a background profile was determined for each sucrose concentration, with the sample cell filled with the sucrose solution in which each sample had been suspended. The electron densities in electrons/Å³ were 0.335, 0.346, 0.359, 0.372, and 0.389 for the 0, 10, 20, 30, and 40.4% sucrose solutions, respectively.

The contribution of the protein-lipid correlation to the total intensity is determined by first calculating the appropriate distance distribution function, $D_{PL}(r)$, from a model of a unit of composition. The geometric model used to calculate $D_{PL}(r)$ is described in cylindrical coordinates (Fig. 1). The electron density of the cylindrical protein, ρ_p , is uniform, while the electron density of the lipid bilayer, ρ_L , is a symmetrical step function of position along the normal to the bilayer. Thus, the number of electron pairs separated by distances between r and $r + dr$ equals the product of electron densities and volume elements integrated over both the volume of the cylindrical protein and the volume of the lipid annulus surrounding it. Taking advantage of the cylindrical symmetry of the model and expressing the position of the annular volume element in relative coordinates, the resulting fivefold integral can be reduced to a single integral and evaluated numerically with a Simpson's rule algorithm implemented on a PDP-11/45 computer. For a detailed discussion

FIGURE 1 (a) The cylindrical protein of height $H_0 + H_n$ radius d , and electron density ρ_p traverses an annular lipid bilayer of half-thickness t and radius D . The electron density of the bilayer is approximated by a step function which takes the value ρ_L^+ in the polar region and ρ_L^- in the hydrocarbon region, which has a half-thickness of t_1 . A correlation at a distance r is indicated between a point (R_c, z_c, ϕ_c) in the protein and a point in the bilayer with coordinate (R, z, ϕ) (taking the first point as the origin). For each r , z and R are mutually dependent; and we have chosen to integrate with respect to z . The integration with respect to ϕ_c is straightforward, but the limits of integration with respect to ϕ depend on the value of R (Fig. 1b). (b) This cross section of the model taken through the point (R, z, ϕ) in the bilayer shows two examples of paths of integration (dashed arcs) with respect to ϕ at radii R_1 and R_2 , from point $(R_c, z_c - z, \phi_c)$. At radius R_1 , the path intersects the cylinder at angle α_d . At radius R_2 , the path intersects the outer boundary of the annulus at angle $\pi - \alpha_d$. These angles of intersection represent the limits of integration with respect to the variable ϕ in half the plane. Thus, the integration over ϕ yields $2(\alpha_d - \alpha_p)$. The angles of intersection, however, depend on the values of R and R_c . In general, $\alpha_d = \arccos [(d^2 - R^2 - R_c^2)/2RR_c]$ when $R < R_c + d$, with a particular value, $\alpha_d = \pi$ when $R \geq R_c + d$. Similarly, $\alpha_p = \arccos [(D^2 - R^2 - R_c^2)/2RR_c]$ when $R > D - R_c$, and $\alpha_p = \pi$ when $R \leq D - R_c$.

of this integral and its derivation, the reader is referred to the Appendix of the previous paper in this series (Brady et al., 1981a). The asymmetry of placement of the protein in the bilayer (the ratio of distances from the ends of the cylinder to the bilayer center) was determined previously to be 4, by fitting this model to the protein-lipid interaction term determined by the method of compositional variation (Brady et al., 1981a). The protein dimensions were also shown to be 142 Å in length and 35.5 Å in diameter. With these parameters, the distribution function was determined at the electron densities of buffers of each sucrose concentration used in these experiments. The corresponding intensity profiles were calculated with the transform

$$I_{PL}(s) = \int_0^\infty D_{PL}(r) \sin sr / sr \cdot dr,$$

evaluated with a Simpson's rule algorithm implemented on a PDP-8/E computer. The error was <1% for the number of points used.

THEORY

Biological membranes are heterogeneous systems consisting of two chemically distinct components, lipid and protein. The intensity of small-angle scattering from a dilute, random suspension of membranes in solvent is proportional to the sum of the independent contributions of the protein and the lipid, and to that of the protein-lipid interaction. Each contribution is concentration-weighted (Brady et al., 1979a):

$$I(s) \sim xI_P(s) + (1 - x)I_L(s) + 2x(1 - x)I_{PL}(s), \quad (1)$$

where x is the mole fraction of protein in the system, and $s = 4\pi/\lambda \sin \theta$ ($\theta = 1/2$ the scattering angle). I_P , I_L , and I_{PL} are the protein, lipid, and protein-lipid interaction contributions, respectively. It is possible to prepare a series of at least three membrane samples that differ in the relative amounts of protein and lipid, and thus the components can be isolated. Each component can be related to structural information in real space by a pair of inverse transforms:

$$D(r) = r \int_0^\infty s I(s) \sin sr \, ds, \quad (2a)$$

$$I(s) = \int_0^\infty D(r) \frac{\sin sr}{sr} \, dr. \quad (2b)$$

The function $D(r)$ is the electron pair distribution function. It gives the number of electron pairs in the system that are separated by the distance r , and represents the total structural information that can be obtained from a small-angle scattering experiment. This function, like the total intensity, is the sum of lipid, protein, and protein-lipid components, each of which can be derived from isolated components of the intensity according to Eq. 2a. Thus, structural information regarding the membrane protein and lipid components as well as their structural interrelationships may be derived from an appropriate series of three or more experiments. As noted in the Introduction, this method of compositional variation was used to isolate the three contributions to scattering from a series of SR membrane suspensions.

This paper confirms those results using samples of SR of identical composition and concentration suspended in a series of buffers of differing electron density. A theoretical explanation for the behavior of the scattering components in such a series of experiments begins with the relationship between the electron pair distribution function and the disparate electron densities within the membrane. If we consider only those variations in electron density of sufficient size to cause scattering in the observable small-angle region, three regions of electron density must be considered: the protein-electron density and the electron densities of the phospholipid polar and hydrocarbon regions. These three regions, each correlating with itself and the other two regions, give rise to six possible scattering correlations. Each correlation is characterized by a point-

pair correlation function, $C_{ij}(r)$, a purely geometric function giving the probability (normalized so that $\int_0^\infty C(r) \, dr = 1$) that two points separated by a distance r contribute to the correlation function of regions i and j . This function is related to its electron pair distribution function as follows:

$$D_{ij}(r) = V_i V_j (\rho_i - \rho_0)(\rho_j - \rho_0) C_{ij}(r), \quad (3)$$

where the subscripts refer to the regions interacting, and V_i and V_j are the volumes of the i th and j th regions, respectively. Because ρ_i and ρ_j are essentially fluctuations about the electron density of the solvent, ρ_0 , the latter must be subtracted from the former to account for the observed small-angle diffraction (see Brady et al., 1979b, for a more detailed explanation). The total intensity scattered by a membrane system in a solvent of electron density ρ_0 is proportional to the sum of the transforms of the six distribution functions:

$$I(s) = \sum_{i=1}^3 \sum_{j=1}^3 V_i V_j (\rho_i - \rho_0)(\rho_j - \rho_0) \int_0^\infty C_{ij}(r) \frac{\sin sr}{sr} \, dr. \quad (4)$$

Since $D_{ij}(r) = D_{ji}(r)$, each interaction between differing regions will be weighted by a factor of two in the remainder of this development (and in third term of Eq. 1).

Each of the component intensities in Eq. 1 is represented in the six terms of Eq. 4. We shall now consider each of these terms, beginning with the protein term. The protein molecule is an extended region of homogeneous electron density, ρ_1 . This means that the scattering intensity due to this component possesses a maximum at $s = 0$ and approximates a reciprocal exponential function of s (Guinier and Fournet, 1955) at low angles. Furthermore, the protein is represented by a single correlation function:

$$I_P(s) = V_1^2 (\rho_1 - \rho_0)^2 \int_0^\infty C_{11}(r) \frac{\sin sr}{sr} \, dr, \quad (5)$$

where V_1 is the volume of the protein component.

The protein component is proportional to the square of the effective protein density. Since the electron density of the protein is higher than the electron density of a dilute buffer (0.400 vs. 0.335 electrons/Å³, in these studies), the protein contribution to the observed scattering diminishes markedly as solvent density is raised by addition of a contrast agent to the solvent. (The protein contribution disappears when $\rho_0 = \rho_1$). Thus, if the protein scattering curve can be determined by fitting a model curve to the experimental intensity at any one solvent density, it can easily be scaled according to the squares of the net protein densities at all solvent densities and subtracted from the experimental curves to yield the sums of the protein-lipid and lipid contributions.

The behavior predicted for the protein-lipid and lipid terms, as solvent density is varied, is more complex than

the behavior of the protein term because the lipid component consists of two dissimilar regions of electron density, i.e., the phospholipid polar group and hydrocarbon regions. Nevertheless, these behaviors may be predicted if we recognize that the electron densities of the two lipid regions are the sum of a volume-weighted mean lipid density, $\bar{\rho}$, and fluctuations, δ_2 and δ_3 , about that mean value, i.e.,

$$\rho_2 = \bar{\rho} + \delta_2, \quad (6a)$$

$$\rho_3 = \bar{\rho} + \delta_3. \quad (6b)$$

Since δ_2 and δ_3 are fluctuations about a volume-weighted average density,

$$\bar{v}_2 \delta_2 + \bar{v}_3 \delta_3 = 0, \quad (6c)$$

where $\bar{v}_2$ and $\bar{v}_3$ are the volume fraction of the membrane lipid occupied by the head group and hydrocarbon regions, respectively.

The distribution function of the lipid, D_L , is the sum of three contributions (Eq. 3):

$$D_L(r) = V_L^2 [\bar{v}_2^2 (\rho_2 - \rho_0)^2 C_{22}(r) + \bar{v}_3^2 (\rho_3 - \rho_0)^2 C_{33}(r) + 2\bar{v}_2\bar{v}_3 (\rho_2 - \rho_0)(\rho_3 - \rho_0) C_{23}(r)],$$

where V_L is the volume of the lipid component. Substituting from Eqs. 6a and 6b for ρ_2 and ρ_3 and rearranging reduces this expression to the sum of three terms that differ in respect to their dependence on $\bar{\rho}$, δ_2 , and δ_3 :

$$D_L(r) = D_{\bar{\rho}}(r) + 2 D_{\bar{\rho}\delta}(r) + D_{\delta}(r), \quad (7)$$

where

$$D_{\bar{\rho}}(r) = V_L^2 \{(\bar{\rho} - \rho_0)^2 [\bar{v}_2^2 C_{22}(r) + \bar{v}_3^2 C_{33}(r) + 2\bar{v}_2\bar{v}_3 C_{23}(r)]\}, \quad (8a)$$

$$D_{\bar{\rho}\delta}(r) = V_L^2 \{2(\bar{\rho} - \rho_0) [\delta_2\bar{v}_2 [\bar{v}_2 C_{22}(r) + \bar{v}_3 C_{23}(r)] + \delta_3\bar{v}_3 [\bar{v}_3 C_{33}(r) + \bar{v}_2 C_{23}(r)]]\}, \quad (8b)$$

$$D_{\delta}(r) = V_L^2 [\delta_2^2 \bar{v}_2^2 C_{22}(r) + \delta_3^2 \bar{v}_3^2 C_{33}(r) + 2\delta_2\delta_3 \bar{v}_2\bar{v}_3 C_{23}(r)]. \quad (8c)$$

The intensity of scattering from the lipid component is the sum of the transforms of these three distribution functions. Some predictions concerning the form- and contrast-dependence of the lipid scattering can be made from an examination of these terms. The transform of the mean density-dependent term describes the scattering from an object of extended, uniform electron density, and is proportional to $(\rho - \rho_0)^2$, with a maximum value at the origin, $I(0) = (\rho - \rho_0)^2 V_L^2$. The mixed term $D_{\bar{\rho}\delta}(r)$ depends linearly on the mean lipid contrast $(\bar{\rho} - \rho_0)$. Thus both these terms vanish at all values of s , including $s = 0$, when the density of the solvent matches the average lipid density. For most phospholipids this is approximately true when

$\rho_0 \approx 0.33$, the electron density of water and dilute buffers. For these solvents, the entire lipid scattering is thus given by the fluctuation-dependent term. This is confirmed by the measurements of small-angle scattering from pure lipid suspensions (Wilkins et al., 1971). These measurements also show that the scattering approaches zero at the origin, a consequence of the fact that the term is proportional to the total content of the fluctuations, which by Eq. 6c is zero for $s = 0$. This contribution is invariant to changes in solvent density, but the two other contributions, especially the first, become more prominent. At high solvent density the mean-density dependent term obscures the other two terms. These predictions were confirmed by calculations of scattering from model lipid bilayers as well as the experimental data (see Results and Brady et al., 1979b; 1981b).

Properties of the protein-lipid interaction contribution can be predicted with a similar treatment. The distribution function for this scattering interaction is the sum of the interactions of the protein electrons with the electrons of the head-group and hydrocarbon regions of the lipid:

$$D_{PL}(r) = D_{12}(r) + D_{13}(r) \\ = V_1(\rho_1 - \rho_0)V_L[\bar{v}_2(\rho_2 - \rho_0)C_{12} + \bar{v}_3(\rho_3 - \rho_0)C_{13}(r)].$$

One sees immediately that this contribution vanishes when the solvent density matches that of the protein. Its dependence on the lipid contrast can be seen if one substitutes for ρ_2 and ρ_3 as before. Then,

$$D_{PL} = V_1 V_L (\rho_1 - \rho_0) \{(\bar{\rho} - \rho_0) [\bar{v}_2 C_{12}(r) + \bar{v}_3 C_{13}(r)] + [\delta_2 \bar{v}_2 C_{12}(r) + \delta_3 \bar{v}_3 C_{13}(r)]\}. \quad (9)$$

Thus, D_{PL} is the sum of two terms, one that depends on the mean lipid electron density, and a second that depends on the fluctuations about that density. When the solvent density matches the mean lipid density, the first term makes no contribution to D_{PL} at any value of s , and the total lipid-protein scattering interaction is determined by the second term. Since its transform (Eq. 2b) is zero at $s = 0$, this term contributes little at the lower values of s , and the net contribution to the scattering is minimal at these angles, for $\bar{\rho} = \rho_0$.

Because the lipid and protein-lipid contributions to the scattering from membranes in dilute buffers are negligible at the innermost range of scattering angles observed in these experiments, the protein term may be derived directly from the experimental data in this region of s . The protein contributions in the other solvents are then scaled from this one curve. The lipid contribution is evaluated by subtracting ρ_0 from the electron density profile of the bilayer shown in Fig. 1 (the model deduced in Brady et al., 1981a), and convoluting to obtain $\tilde{\rho}_L^2$ at each value of ρ_0 . Fourier transformation then gives I_L . Subtracting the protein and lipid terms from the experimental curves then

isolates the protein-lipid contribution at each value of ρ_0 , which can then be compared with the same term derived in the previous study for $\rho_0 = 0.335$, and then scaled to the other values of ρ_0 as described under Methods. The agreement provides an independent test of the validity of the earlier determination of the protein dimensions and its location in the bilayer, since both of these aspects of the model derived therein enter into the evaluation of its I_{PL} .

RESULTS AND DISCUSSION

With the preceding theoretical presentation as background, the analysis of the experimental results is straightforward. The scattering curves as a function of solvent electron density are shown in Fig. 2. The first qualitative feature to be noted is the intense halo around the origin. This scattering appears to suffer a slight decrease as the solvent density is first increased, and then shows an increase at the higher densities. The second feature is the behavior of the broad band at $s \approx 0.12$, previously identified (Brady et al., 1981a) with the lipid-protein scattering interaction. Here again the trend with varying solvent density is not clearcut. The last feature concerns the appearance of that portion of the curves beyond $s \approx 0.15$. The scattering in this region arises primarily from the lipid component. At the lowest electron density, it is characterized by a peak that appears as a shoulder on the right-hand side of the lipid-protein interaction peak. With increasing solvent density, it fades out, transforming at the highest

density into a tail on the rest of the scattering curve. As there is obviously an extensive overlap of the three components in some regions of the curves, it is not worthwhile at this stage to continue the description further. The trends will become much more apparent after the curves are decomposed into their three components.

We start the analysis with the scattering at the innermost angles. As described in the previous section, the intensity in this region for the membrane in dilute buffer ($\rho_0 = 0.335$) consists overwhelmingly of the independent protein scattering. This results from the fact that at this value of ρ_0 , the contributions of the excess positive polar head group electron density and the excess negative density of the interior hydrocarbon region largely counterbalance, with the result that the lipid scattering contribution becomes very small at the low angles (Brady et al., 1981a). This counterbalancing, however, is no longer operative when the solvent electron density is changed to another value, and at the other values of ρ_0 the inner scattering will include considerable lipid scattering. Thus the protein scattering can only be isolated in the dilute buffer (curve 1, Fig. 2). Its contribution at the other values of ρ_0 is then obtained by scaling this curve to these values. As shown in Eq. 5, the scaling factor is $(\rho_1 - \rho_0)^2$. The resultant protein curves are shown by the dashed lines in Fig. 2. Subtracting these from the total scattering curves then gives curves which represent the sum of the scattering contribution of the lipid and lipid-protein terms of Eq. 1. These are shown in Fig. 3. Since the protein scattering was considered at length in the previous paper (Brady et al., 1981a), the rest of the discussion will be concerned with identifying and isolating the separate contributions of these two remaining components to the curves in Fig. 3.

FIGURE 2 Measured scattering curves of sarcoplasmic reticulum membrane at different values of ρ_0 , the solvent electron density. The curve for $\rho_0 = 0.372$ has been left out but reappears in the subsequent figures. The dashed curves are the protein scattering components for the three values of ρ_0 indicated.

FIGURE 3 The curves of Fig. 2 with the protein scattering removed. They represent the sum of the lipid and lipid-protein terms of Eq. 1. The intensity values in the ordinate apply to curve 5. The ordinate origin for curves 4, 3, 2, and 1 is shifted downward by successive decrements of $\Delta I = -250$; thus the ordinate values for these curves should be increased accordingly.

The contribution of the lipid component in the dilute buffer solvent has been identified as a relatively weak band centered at $s = 0.182$ and tailing off to negligible values at higher s (Brady et al., 1981a). The low magnitude of the lipid scattering resulted from the fact that a membrane composition with a high mole fraction of protein had been chosen to accentuate the relative contributions of the protein and protein-lipid terms. This was done, of course, at the expense of the lipid mole fraction, with the result that its relative contribution was too small to be measured exactly. Fortunately, the lipid scattering increases with increasing solvent electron density to a point where it constitutes a major component of the scattering even at this mole fraction of lipid. This follows directly from consideration of Eqs. 7 and 8a-c, where the dependence on net lipid electron density contrast $(\bar{\rho}_0 - \rho)^2$ is clearly evident; at the same time, Eq. 7 shows that the magnitude of the protein scattering decreases. To calculate the lipid contribution, we show the lipid bilayer in Fig. 4 first as a rectangular distribution of positive and negative electron density. The positive regions are 10-Å thick and the distance between their centers is 41 Å. Then, in model II, the structure has been rendered more factual by substituting approximately Gaussian distributions of the same area for the rectangular regions. The highest (0.387) and lowest (0.335) solvent electron densities are represented as horizontal lines drawn through the profiles. Rather than

FIGURE 4 Scattering curves for the membrane lipid bilayer as a function of ρ_0 . As described in the text, the rectangular model (I) in the inset is smoothed out to give the electron density distribution (II), keeping the respective total areas of the positive and negative regions the same. The values of $\rho_- = 0.270$ and $\rho_+ = 0.390$ were used for the electron densities of the interior hydrocarbon and polar head group regions, respectively. The corresponding convolutions of the electron density profiles for the highest and lowest values of ρ_0 are also shown in the inset. The points at the right end of the $\rho_0 = 0.335$ intensity curves are the measured values.

evaluate the separate components of Eqs. 8a-c, it is convenient and more instructive to evaluate their total. This is done directly by evaluating the double sum

$$\sum_{r_2} \sum_{r_3} [\rho_2(r) - \rho_0][\rho_3(r) - \rho_0],$$

where $(\rho_2 - \rho_0)$ and $(\rho_3 - \rho_0)$ are the effective electron densities of the two regions of the lipid. This is easily done on a computer. The double sum is the convolution, $\hat{\rho}_L^2$, and is the Fourier transform of the lipid scattered intensity. It is shown in the inset of Fig. 4 (multiplied by r) for $\rho_0 = 0.387$ and $\rho_0 = 0.335$. The intensities, obtained by inverse Fourier transformation are shown for the three values of ρ_0 indicated on the graph. The curves illustrate the marked effect of increasing ρ_0 . As the peak at $s = 0.18$ fades out, a strong scattering develops, which intensifies toward the lower values of s .

The open circles seen to the right of the $\rho_0 = 0.335$ curve are the experimental values of the scattering at this solvent density. These were used to normalize the calculated intensity curves to the same units as the experimental ones by scaling to the peak maximum at $s \approx 0.18$. In addition, this curve was used to iteratively adjust the widths of the Gaussian in the bilayer profile so that its corresponding intensity curve matched as closely as possible the right-hand side of the lipid peak (at $s = 0.18$), where the scattering in the measured curve is essentially free of any contribution from the lipid-protein component. This procedure insured that the calculated lipid curves at the lower values of ρ_0 represented as closely as possible the lipid component contained in the experimental curves. Moreover, it turns out that the intensity curves are sensitive to the details of the distribution curve only at the lower values of ρ_0 , where the net difference between the effective densities $(\rho_2 - \rho_0)$ and $(\rho_3 - \rho_0)$ is small. At higher ρ_0 , where this difference becomes significant owing to the simultaneous increase and decrease of the effective negative and positive densities, the intensity curves are determined principally by the difference in total charge density between the positive and negative regions.

The values of the lipid-protein scattering interaction term (the second component of the curves in Fig. 3), isolated from the membrane scattering in dilute buffer by the use of the composition variation method (Brady et al., 1981a), are shown as curve 1 in Fig. 5. Their Fourier transform D_{PL} reduces at this solvent density to a term that is determined by the product of the protein electron contrast $(\rho_1 - \rho_0)$ and a quantity that depends on the fluctuations in density of the polar head and hydrocarbon regions of the lipid about its mean density $\bar{\rho}$. In part I of this paper (Brady et al. 1981a), the correlation terms C_{12} and C_{13} had been evaluated by computing D_{PL} for various positions of the protein in the bilayer, and then comparing the results with its experimentally determined value. We now use these terms with Eq. 9 to evaluate the D_{PL} corresponding to the other values of ρ_0 ; all the other

FIGURE 5 The lipid-protein scattering contribution curve 1 was obtained previously by use of the concentration-variation method. Curves 2–5 are thus curve 1 scaled to the other values of ρ_0 by the method described in the text.

variables in the equation are known. Fourier transformation of the D_{PL} then gives the corresponding intensities, I_{PL} (Fig. 5), which show an increasing rate of decrease with increasing ρ_0 , reflecting the simultaneous influence of the factors $(\rho_1 - \rho_0)$ and $(\bar{\rho} - \rho_0)$ in Eq. 9.

The scaled curves of Figs. 4 and 5, added together for each value of ρ_0 , can now be compared to the experimental curves of Fig. 3 (Fig. 6). The points are the experimental values. The agreement is very good throughout the whole range of s except for the innermost values of s , where the experimental curves show a sharp upward deviation, which becomes more intense with increasing solvent electron density. This excess scattering will be discussed presently. The fading out of the peak at $s = 0.18$ and the shift of the bulk of the intensity inwards is paralleled in both sets of curves. The sum of the I_L and I_{PL} components scaled from the earlier model is in agreement with the experimental curves seen in Fig. 7, where we compare I_{PL} (determined by subtracting the calculated I_L curves from the experimental curves in Fig. 6) with the scaled I_{PL} values in Fig. 5.

FIGURE 6 Comparison of experimental (Fig. 3) and calculated (Figs. 4, 5) lipid and lipid-protein contributions. The experimental values are the points. The ordinate values for curves 4, 3, 2, and 1 are to be adjusted as described in Fig. 3.

FIGURE 7 Comparison of scaled values (Fig. 5) of the lipid-protein term with those determined by subtracting the calculated lipid contributions (Fig. 4) from the experimental curves (Fig. 3). The ordinate origin for curves 1–4 is shifted downwards by successive decrements of $\Delta I = -100$; the ordinate values for these curves should be increased accordingly.

Once again the agreement is very good, which confirms our previous determination of the protein shape and dimensions and its placement in the bilayer. It should be noted that the criterion used previously to determine the placement of the protein was the agreement between the experimental maximum of the lipid-protein interaction and the maximum computed as a function of the asymmetry of the protein position with respect to the bilayer center. Here the criterion is the agreement of I_{PL} at all values of s , obviously a more rigorous requirement.

The excess scattering at low s (Fig. 5), obtained by subtracting the background lipid curves, is shown in Fig. 8 for the three highest values of ρ . The curves have been

FIGURE 8 Curves of the excess scattering at the inner angles in Fig. 3. The curves are for the three highest values of ρ_0 . The mean of the three curves has been replotted in the lower portion of the figure. This portion also shows calculated intensity curves for a spherical [S] and disklike [D] electron density distribution (see text).

normalized to unity at $s = 0$ by dividing by the ordinate intercepts obtained from extrapolation of a $\ln I$ vs. s^2 plot. The figure shows that within experimental error the points superimpose except perhaps at the lower values of s , where the differences from underground are small. This demonstrates that the form of this correlation does not change with increasing solvent density. The fact that the intensity is confined to the smallest angles and increases rapidly in intensity with decreasing angle indicates that the correlation is long-range. In this region, around the origin, the intensity is proportional to $|(\rho_2 - \rho_0) + (\rho_3 - \rho_0)|^2$, and can be written as

$$I \sim |(\rho_2 - \rho_0) + (\rho_3 - \rho_0)|^2 \int_r C(r) \frac{\sin sr}{sr} dr,$$

where $C(r)$ is a correlation function that decreases from unity at $s = 0$ and tends toward 0 for large r . Obviously only the outer part of the correlation is seen and ideally the measurements should have been extended into lower values of s , but this was not possible with the present equipment. But, a sufficient range has been covered to allow some significant conclusions about the form of the correlation to be drawn. In the lower portion of Fig. 8, intensity curves corresponding to correlation functions for spherical and disklike distributions of electron density are shown. The curves have been calculated for nearly equivalent radii of gyration (or correlation lengths), chosen so that the intensities tended to a negligible value in essentially the same region of s as the experimental ones. The spherical scattering curve corresponds to that from a sphere of 55-Å radius. The ratio of thickness to diameter of the disklike distribution (of radius of gyration of 38 Å) is $\sim 1/4.5$). A thickness of 35 Å, a reasonable value for the effective bilayer thickness of the lipid at these solvent densities, would dictate a value of ~ 158 Å for the electron distribution in the direction normal to the bilayer. In this angular range the scattering for a distribution of this form is given by (Guinier and Fournet, 1955)

$$I \sim \frac{1 - \nu^2 L^2 s^2}{s^2 L^2},$$

where ν is the ratio of the two dimensions, and L is the larger dimension. Significantly, the intensity decreases principally as s^{-2} . The averaged scattering of the three experimental curves, normalized to the same scale as the calculated curve, is replotted on this portion of the graph also. Examination shows that the excess scattering follows the curve for the flat distribution over almost the whole scattering range, deviating only at the higher end where the intensities approach background level. The data are incompatible with either spherical or cigar-shaped distributions.

Since the protein and transbilayer lipid scattering have been accounted for, the only remaining source of the excess scattering is the in-plane correlation of the bilayer

FIGURE 9 Schematic of the sarcoplasmic reticulum membrane. The calculation outlined in Fig. 1, when compared with the experimental data, indicates that one end of the protein molecule is contiguous with a bilayer face.

electrons. This is consistent with the high value of the correlation length and with the dimensions and asymmetry of the correlations with respect to one of the three directions. Physically what is being measured are the mean dimensions of the bilayer plane. For a flat continuous surface L would approach infinity, and the scattering would be confined to immeasurably small angles. In actuality, thermal fluctuations and diffusional motion, along with disruption and interaction with penetrating protein, modify it into a wavy sheet extending preponderantly in two directions, but showing significant curvature at large distances. The significant range of L , however, shows that the characteristic two-dimensional lipid structure is maintained at the relatively low lipid-to-protein ratio used in the present study. Therefore, its representation as a disc of these dimensions seems a reasonable one.

To summarize, our results are in agreement with those of the previous work on the sarcoplasmic reticulum membrane, and confirm that the protein-lipid configuration deduced earlier (Fig. 1) is essentially correct. In addition, the electron contrast variation measurements have allowed us to identify the lipid component of the scattering, and from it to evaluate the electron density profile of the latter. The technique has also enabled us to isolate the in-plane component of the lipid scattering. With this added information the membrane analysis is complete. Perhaps of equal importance is the fact that the work illustrates how the relatively straightforward application of theory to a series of measurements at different solvent densities allows us to resolve the scattering components. In so doing, insight is gained into the scattering process, allowing for future extension to other systems. Since the method is based on the addition and subtraction of different scattering components, the intensity measurements must be precise, but this can easily be accomplished to the required degree of accuracy with a position-sensitive detector.

Our results can be compared with some electron micro-

scopy observations. These are essentially qualitative, but useful agreements emerge. Stewart and MacLennan (1974) noted that in negatively stained SR vesicles, particles with a diameter of 30–40 Å are removed by trypsin digestion with a concomitant loss of Ca^{++} -ATPase activity. An alignment of protein, finding the cytoplasmic face of SR has been deduced from staining studies on thin sections of isolated membranes (Saito et al., 1978). These findings agree with ours, as do the indications from sedimentation behavior of an active complex of detergents, phospholipid, and monomeric ATPase, that the latter is an elongated molecule (LeMaire et al., 1976a). X-ray studies of stacked multilamellar assemblies of SR membranes (Dupont et al., 1973; Liu and Worthington 1974; Herbet et al., 1978), although not particularly successful in locating the protein (owing, among other things, to the necessity of estimating a background line around which to base the ($\pm$) phase assignments, and to ambiguities on these assignments), have yielded electron density profiles of the system. These disagree to some extent in the details, presumably because of the above factors, but all show an asymmetric density distribution, which has been rationalized as indicating an asymmetric placement of the protein across the bilayer.

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