Measurements of Membrane Thickness by Small-Angle Neutron Scattering of Suspensions: Results for Reconstituted *Rhodopseudomonas sphaeroides* Reaction-Center Protein and for Lipids†

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ABSTRACT: Small-angle neutron scattering has been used to measure thicknesses of membranes in suspension. Results are presented for large single-walled vesicles of lipids and of lipids reconstituted with a membrane protein (reaction centers from the photosynthetic bacterium *Rhodopseudomonas sphaeroides*). Special methods of sample preparation were used, and the detailed characterization of the preparations is described and discussed. The conclusion is that the protein does not project far from the lipid layer; if a simple cylindrical model is used, its transmembrane length would be about 52 Å. The results are compared with similar ones obtained for the intact photosynthetic membranes, containing reaction centers as well as other proteins; they are discussed in line of previous studies on diffraction of stacks of lecithin bilayers containing reaction centers reconstituted in a different way.

The purpose of this work is to develop methods of lipid/protein reconstitution and then to exploit them in order to investigate the molecular structure of the membranes by small-angle neutron scattering from unilamellar suspensions (Sadler & Worcester, 1982). Neutron scattering, because it relies on the interference phenomenon such as in diffraction, can measure some of the features of the structure at low resolution, such as the extent to which proteins project from the lipid part of the membrane. The system studied contains the bacterial reaction-center protein (RCP)[1] isolated from *Rhodopseudomonas sphaeroides* (*R. sphaeroides*) in which the primary processes involving charge separation during photosynthesis take place. This integral membrane protein, isolated by detergent extraction, contains three polypeptide chains and a number of pigments and cofactors: four bacteriochlorophylls, two pheophytins, two ubiquinones, and one iron [for a review, see Feher & Okamura (1978)]. In the intact bacterial membrane, it is asymmetrically oriented with at least two of the polypeptides spanning the bilayer (Valkirs & Feher, 1982).

The method of scattering from membrane suspensions, like many studies of diffraction from membrane stacks, concerns mainly the scattering density distribution as projected on to the lamella normals. The two methods are not equivalent however: some of the general features of the suspension scattering method (Sadler & Worcester, 1982) are as follows. Intensities are measured at small values of s [$s = (2 \sin \theta)/\lambda$, where 2θ is the scattering angle and λ is the wavelength] of no greater than about $^1/_{30}$ Å^{-1}. In the limit of small s, dimensions (thicknesses) can be obtained almost independently of any model (with the Guinier formula). By measurement of the way thickness depends on the scattering properties of the medium, with $^1H_2O/^2H_2O$ mixtures, a thickness can be derived that depends only on those regions that displace the medium and not on the internal details of the membrane. The thickness values derive from a weighted mean of z^2, where z is the distance from the center of the membrane along the normal. Hence, the method is especially sensitive to features at high z due to, for example, proteins extending out beyond the layer associated with a lipid bilayer.

The first application of the technique was to photosynthetic membranes obtained from bacteria. Under the experimental conditions used, the RCP and the light harvesting proteins together represent about three-fourths of the protein in the membrane. It was shown that the membrane was not very much thicker than that expected for a lipid layer (40 Å). For the simplest type of model with an "average" protein consisting of cylinders projecting through a lipid layer, the total length of the cylinder would be 54 Å (Sadler & Worcester, 1982).

It is now possible to perform a similar experiment with just the RCP present. It will be shown that its total length (again using a simple cylinder model) is 52–54 Å, i.e., slightly less than the value for the average. This result will be compared with results of diffraction from stacks of reconstituted membranes, which gives complementary information (Pachence et al., 1979, 1981, 1983; Blasie et al., 1983).

The methods of membrane preparation have been chosen to optimize the use of the scattering technique. It is clearly important to use single-layer vesicles and, preferably, those with relatively large radii of curvature, since the scattering is much more easily interpreted in the absence of well-defined curvatures (Sadler & Worcester, 1982). This excludes the most common lipid preparations, which are either multilamellar or very small sonicated vesicles.

Materials and Methods

Chromatographically homogeneous egg phosphatidylcholine was prepared by the method of Singleton et al. (1965); chemically pure dodecyldimethylamine oxide (DDAO) was obtained from the CEA (Saclay, France) in its cold and labeled ([^{14}C]DDAO, 24 mCi/nmol) forms.

Wild-type (strain Y) *R. sphaeroides* cells were grown anaerobically in light in the "L 17 μM Fe" medium (Reiss-Husson et al., 1971). RCP were isolated with LDAO and purified as described by Jolchine & Reiss-Husson (1974) with an additional purification by DEAE-cellulose with a linear NaCl gradient. RCP preparations were finally concentrated by ultrafiltration under N_2 on an Amicon PM 30 membrane

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[1] Abbreviations: RCP, reaction-center protein; LDAO, commercial lauryldimethylamine oxide; DDAO, dodecyldimethylamine oxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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(up to 4 mg of protein/mL) in a 10 mM Tris-HCl–0.1 mM EDTA buffer, pH 8.0, in presence of 1 mg of LDAO/mL.

Phosphorus content was determined by the method of Bartlett (1959) and lecithin calculated with a multiplying factor of 25. Protein concentration was routinely measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Standard curves were always run in the same buffer including detergent as those used for the reaction-center samples.

Preparation of Lecithin Vesicles. Several techniques were employed for the preparation of lipid liposomes of various sizes. (1) Sonicated lecithin vesicles. Lecithin was dried under nitrogen and suspended in buffer at a concentration of approximately 50 mg/mL and shaken for 5 min in a Vortex, in a 10 mM Tris–0.1 mM EDTA buffer, pH 8.0, which was used throughout this work. It was then ultrasonically irradiated and treated as described by Huang (1969). (2) Reverse phase evaporation vesicles (REV). They were prepared as described by Szoka et al. (1978), then filtered on a polycarbonate Unipore Bio-Rad membrane (pore size 0.4 μm) for the elimination of big multilayer liposomes, and finally fractionated by chromatography as described under Characterization.

Preparation of Proteoliposomes. RCP solutions in LDAO (1 mg/mL) were used for incorporation into sonicated lecithin vesicles prepared as described above. In a typical experiment, 1 volume of RCP solution was added with shaking to a liposome suspension (10 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, pH 8.0) to give a protein to lecithin ratio of 30% (w/w) and a final concentration of protein lower than 2.0 mg/mL. The incorporation of RCP into the liposomes was followed by an instantaneous increase of the turbidity of the mixture. Reconstituted proteoliposomes were separated from the lecithin–detergent mixed micelles by molecular sieve chromatography on a Sepharose CL-4B column, previously saturated with the lecithin dispersion and then equilibrated with the buffer solution so as to eliminate lipid adsorption (Huang, 1969). All fractions eluted from the column were analyzed for lipids and proteins. Reconstituted proteoliposomes were concentrated on a YM 10 hydrophobic membrane (Amicon) up to about 3.5 mg of protein/mL and 9.4 mg of lecithin/mL. Control experiments were run with phosphatidylcholine vesicles alone.

Characterization. A column of Bio-Gel A-150m (mesh size 100–200) (1.6-cm diameter × 62.5-cm height) was employed for determining the size distribution of the various vesicle preparations. The column was equilibrated at 4 °C with the buffer solution; 10 mL of vesicles suspension was deposited on the top and eluted. Fractions of 0.8 mL were collected. The column was finally calibrated (Rhoden & Goldin, 1979) with latex particles (Dow Chemical Co.) and preequilibrated and eluted with buffer containing 1% Triton X-100, to prevent aggregation of latex beads. Under these conditions, this gel did not cause aggregation of lipid vesicles. Nevertheless when Bio-Gel A-150m of mesh size 50–100 was employed, we observed extensive aggregation, as already reported by Chen & Schullery (1979).

Two different experiments were made to determine the amount of detergent that remains bound to RCP after reconstitution. In the first, 1 volume of RCP preparation in LDAO (1 mg/mL) was dialyzed against 480 volumes of TL buffer (10 mM Tris, 1 mM EDTA, 1 mg/mL radioactive DDAO, specific radioactivity 11.45 μCi/mmol) during 28 h. The dialysis buffer was changed once during the night. A aliquot of this RCP solution in radioactive DDAO was added with shaking to 1.0 mL of liposome suspension in buffer to give a protein to lecithin ratio of 8.6% and a concentration of 1.36 mg of protein/mL. Reconstituted liposomes were isolated by molecular sieve chromatography as described before. All fractions eluted from the column were analyzed for protein, phospholipids, and radioactivity. The second experiment was identical except in the way cold LDAO was exchanged for [^{14}C]DDAO; the RCP aliquot was incubated 24 h with 2.5 μCi of [^{14}C]DDAO. A control was done in parallel by incubating 1 mg of DDAO in 1 mL with the same amount of [^{14}C]DDAO.

For freeze–fracture electron microscopy, small drops of preparations, containing 25–30% glycerol, were deposited on conventional Balzers gold planchets and rapidly frozen in Freon 22 (at −160 °C). Fracturing and replication were done with Balzers BAF 301 freeze–etching unit by using platinum–carbon shadowing. The replicas, after digestion of organic material with chromic acid and washing with distilled water, were observed in a Philips 301 electron microscope.

Optical spectra were measured at 25 °C with a Cary 14 R spectrophotometer and an Aminco DW 2 A spectrophotometer, both equipped with a side illumination accessory.

Neutron Scattering Measurements. Measurements were made by taking suspensions at circa 1% dry weight in quartz spectrophotometer cells. Adjustment of the $^1H/^2H$ ratios was achieved by dialyzing part of the preparation in an equivalent medium based on ^2H_2O. Mixtures of this and the ^1H_2O preparation were made with Hamilton syringes the day prior to the scattering experiments. Neutron scattering experiments were made on D11A and D17 instruments at the Institute Laue Langevin (Grenoble) (Schmatz et al., 1974) and on the small-angle spectrometer at the Atomic Energy Authority (Harwell). Wavelengths were 6, 10, or 11 Å, and specimen to detector distances were 4.4, 2.8, 2.1, and 1 m. The s ranges were typically 0.002–0.013 Å^{-1}, and additional short measurements were made up to $s \simeq 0.04$ Å^{-1} in order to check diffuse scattering (in particular, incoherent scattering). Transmissions were monitored systematically. Background runs were made with mixtures of $^1H_2O/^2H_2O$ buffers, occasionally sufficiently long to achieve high statistical accuracy, and these were the spectra used in background subtraction calculations. In other cases, short background runs were made. In this way, a large number of background runs could be avoided. The short runs were used to check the method of scaling of the incoherent scattering in the calculations. The counter response was calculated from the (incoherent) scattering from ^1H_2O.

Analysis of Neutron Scattering. The contrast variation technique (Stuhrman, 1974) is used to separate the effects on the scattering of scattering length density (ρ) differences within the membranes from those due to the surrounding medium. Specifically [as given by Sadler & Worcester (1982)]

$$\rho(z) = \rho_F(z) + \rho_w(z)\bar{\rho} \quad (1)$$

where z is the distance along the lamellar normals and $\bar{\rho}$ is the difference between the mean density of the lamella and that of the medium. $\rho_w(z)$ is dimensionless and is in the range $0 < \rho_w(z) < 1$. Intermediate values can arise as a result of $^2H/^1H$ exchange between membrane components and the medium. For the one-dimensional case, this "exchange" includes the effects of the membrane having nonplanar surfaces (e.g., as a result of projection from the surfaces).

In the limit of small s, the coherent intensity contribution due to the membranes is given by $I(s)$:

$$I(s) = A(\bar{\rho}t)^2 \exp(-4\pi^2 s^2 D^2)/(2\pi s^2) \quad (2)$$

where the "thickness parameter" D is given by

$$D^2 = \int z^2\rho(z)\,dz / \int \rho(z)\,dz \quad (3)$$

A is the total area of membrane in the sample, t is the thickness, which is the one-dimensional analogue of the volume of a globular object given by $t = \int \rho(z)\,dz/\bar{\rho}$. An additional term $I_a(s)$ in eq 2 has been considered previously (Sadler & Worcester, 1982) due to the residual intensity that arises because of scattering length density changes with respect to displacement in the plane of the membranes. No evidence has been found for the existence of an $I_a(s)$ term during the present experiments.

The calibration of the intensity was achieved (Jacrot & Zaccai, 1981) by measuring the count rate for incoherent scattering I_i and calculating the corresponding (macroscopic) cross section from the attenuation $(1 - T_i)$. The ratio of count rate to cross section for the ^1H_2O medium is then known. Hence, the count rate can be converted to a (differential) cross section. For incoherent scattering

$$I_i = \frac{1}{4\pi f}(1 - T_i)S \quad (4)$$

S is the area illuminated by the beam (constant over all measurements), and f is a wavelength-dependent empirical correction factor to allow for effects such as multiple scattering (Sears, 1975) and inelastic scattering (Reinsch, 1961; Beyster, 1968). The sample spectra, after background subtraction, are computed as a ratio of count rates, which can be written as $I(s)/(T_sI_i)$, where T_s is the transmission of the sample. The volume of membranes is At, which is equal to cV, where c is the volume fraction of membranes in the suspension and V the volume of sample illuminated by the beam. Hence

$$I'(s) = \frac{I(s)}{T_sI_i} = \frac{4\bar{\rho}^2 t\pi f V c}{T_s(1 - T_i)S}\,\frac{\exp(-4\pi^2 s^2 D^2)}{2\pi s^2} \quad (5)$$

In order to make the units consistent, V/S (the thickness of the suspension in the quartz cells) is in angstroms, and $\bar{\rho}$ is in units of $Å^2/Å^3$, i.e. $Å^{-1}$.

The hypothesis is now made that there are no significant density differences as a result of "bound" water and that there is no preferential adsorption of protons or deuterons. We then have, as a result of contrast variation formalism

$$D^2 = D_w^2 + \alpha/\bar{\rho} - \beta/\bar{\rho}^2 \quad (6)$$

where

$$D_w^2 = \int z^2\rho_w(z)\,dz/t \quad (7)$$

$$\alpha = \frac{1}{t}\int z^2\rho_F(z)\,dz \quad (8)$$

and

$$\beta = \left[\frac{1}{t}\int z\rho_F(z)\,dz\right]^2 \quad (9)$$

Equation 2 describes accurately most of the data (see below) but is inadequate when $\rho \simeq 0$ (near the "match" point). D is then very large, and the exponential approximation does not hold. Experiments were carried out in this region of contrast in order to check the self-consistency of the analysis and were analyzed as follows.

A model for $\rho(z)$ must be used, and for the purpose of exploring the intensities near the match point, this was a two-level density function. As discussed below, this function does not have a direct interpretation in terms of regions of particular molecular groups. Arbitrarily, $\rho_F(z)$ is approximated by a central layer of thickness $d/2$ and density $-\rho_1$ with layers on the either side of thickness $d/4$ and, hence, density ρ_1. d is chosen such that $d = 2(3)^{1/2}D_w$.

For one uniform membrane

$$I(s) = A\left[\int \rho \exp(2\pi isz)\,dz\right]^2/(2\pi s^2) = \frac{A}{2\pi s^2}\left[\frac{\bar{\rho}d\sin(\pi sd)}{\pi sd} + \frac{\rho_1 d\sin(\pi sd)}{\pi sd} - \frac{2\rho_1(d/2)\sin(\pi sd/2)}{\pi s(d/2)}\right]^2 \quad (10)$$

where the scattering amplitude of a "top-hat function" of width d is given by $[\sin(\pi sd)]/(\pi sd)$. It is easily verified that $I(s)s^2$ goes to zero at small s as required when $\bar{\rho} = 0$. Equation 10 can readily be used to compute the scattering for a heterogeneous preparation where membranes of several $\bar{\rho}$ values are present at the same ^2H_2O content.

Results and Discussion

Characterization of Proteoliposomes. When sonicated liposomes were incubated with RCP and subjected to gel filtration, the elution profile shown in Figure 1A was obtained. The RCP was eluted with most of the lipid in the void volume of the column, suggesting that these vesicles were much bigger than before the incubation. Rechromatography of the void volume fraction did not modify its elution, and it was also eluted in the void volume of a Bio-Gel A-150m column, which has a higher exclusion limit (Figure 1B). Prolonging the incubation overnight did not modify the chromatographic profiles shown in Figure 1A. Control experiments on pure liposomes incubated with LDAO (at a similar concentration) showed that the formation of these large vesicles required the interaction of liposomes and RCP.

The chromatographic behavior of the sample was dependent on its composition. With a given liposome amount (20 mM lecithin), a partial and irreversible precipitation occurred if the protein concentration was higher than 3 mg/mL or the ionic strength was too weak (less than 30 mM NaCl). Precipitation resulted in part of the sample being retained on top of the gel. The protein to lipid ratio changed in general during successive runs, but this could be minimized by saturating the agarose gel with lipid prior to runs with proteoliposomes. Agarose is known to bind lecithin (Huang, 1969). If incubated gels were used, the protein to lipid ratio decreased with elution volume by a factor of up to about 3. Since the fractions were pooled for the scattering experiments, the final samples have a significant heterogeneity. An idea of the degree of heterogeneity was obtained by calculating the root mean square (rms) of the difference in protein to lipid ratios between that for the individual fractions and that for the "pool". For three experiments of this type, the protein to lipid ratios were 0.58 (rms difference 0.18), 0.21 (rms difference 0.1), and 0.22 (rms difference 0.09).

In the proteoliposome fraction, the mean protein to lipid ratio depended on the initial ratio during incubation but was never less than 0.2. If more lipid was used, it was not incorporated in the proteoliposome fraction. Hence, the method could not be used to prepare large vesicles each containing a small number of RCP.

The amount of LDAO eluted in the void volume fraction was very low: respectively, 1.0 and 1.8 molecules of LDAO/RCP was found in two independent experiments (see Materials and Methods) (Figure 1A). This LDAO might be bound to the RCP [compared to over 200 LDAO molecules bound to RCP in detergent solution (Rivas et al., 1980)], or

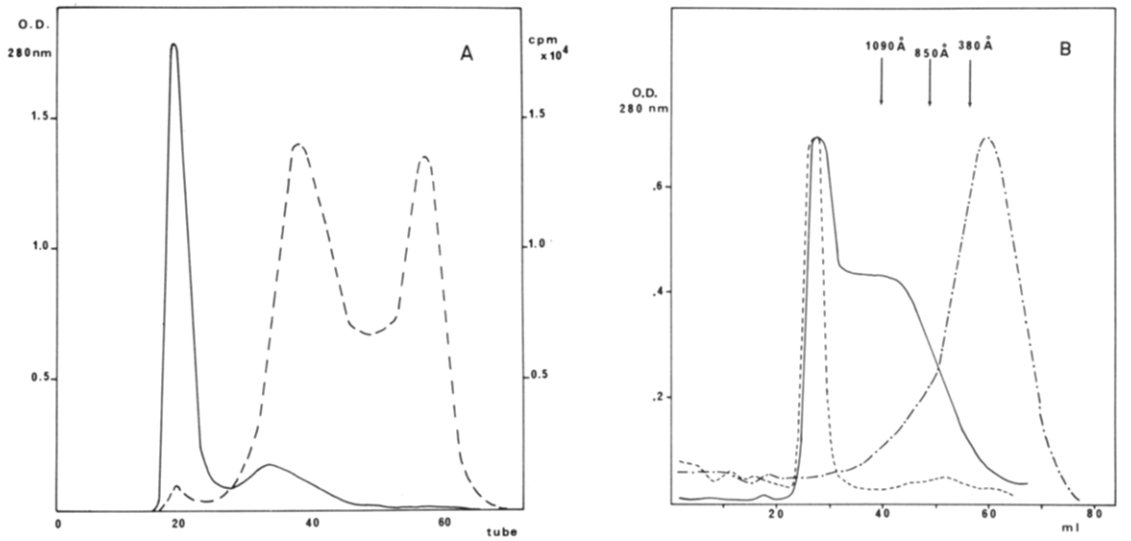

FIGURE 1: (A) Elution profiles of a [^{14}C]DDAO–RCP solution after incubation with sonicated liposomes, chromatographed on a Sepharose CL-4B column (1.6 × 28 cm). (—) The optical density was measured at 280 nm. Reconstituted liposomes eluted in a sharp peak in the void volume. (– –) Radioactivity due to the [^{14}C]DDAO elution (see text). (B) Elution profiles (measured as optical density at 280 nm) of a chromatography on a Bio-Gel A-150m column (1.6 × 32.5 cm) of liposomes prepared by reverse-phase evaporation and filtered on a polycarbonate membrane (—). For neutron experiments, fractions eluted between 23 and 32 mL were pooled. Reconstituted proteoliposomes (– –) (void volume fraction from the Sepharose column, see Figure 1A). Sonicated egg lecithin liposomes used for the reconstitution experiments (–·–). The column was calibrated with latex beads of different diameters (arrows).

it might be associated with the small number of liposomes that were eluted with the proteoliposomes. The rest of the LDAO was retarded by the gel and eluted in two peaks, which were also observed in a control experiment performed with liposomes without RCP.

Numerous proteoliposome preparations and all samples used in the neutron scattering experiments were examined by electron microscopy. They contained unilamellar vesicles, the proportion of the multilamellar one being insignificant. Most of them were proteoliposomes of various sizes, characterized by a uniform distribution of intramembrane particles on fracture faces (Figure 2). The mean diameter of the vesicles was 1900 ± 1000 Å, with a broad distribution. In a preparation with a protein to lipid ratio of 0.57, 3220 ± 1070 particles/μm^2 were observed, that is, one particle per square of 176 × 176 Å.

The vesicular nature of the proteoliposomes could be demonstrated by their capacity to entrap cytochrome *c* (cyt *c*), when the reconstitution was performed with cyt *c* loaded liposomes. The internal volume of the proteoliposomes was measured from the trapped cyt *c* content (5.021 L/mol of lecithin); by adopting 40 Å for the width of the bilayer (see below) and 70 Å^2 for the average interface area per polar group, the average vesicle diameter could be estimated to be 1600 Å, which is consistent with the electron microscopy results.

During illumination, RCP were reversibly photooxidized; under the same illumination but in the presence of cyt *c* added exogenously, they stayed reduced at 90–100%. A photoinduced carotenoid shift similar to that reported for RCP in LDAO solution (Heathcote et al., 1977) was also observed; it was abolished by addition of cyt *c*. On these two criteria, the RCP were oriented with the cytochrome reaction sites available only on the external surface of the vesicles.

The infrared spectrum of the proteoliposomes showed some modifications as compared to that in detergent solutions, although these were absent at 77 K. The maximum of the dimer Q_y band was shifted from 866.5 to 869.5 nm and seemed to be enhanced, in agreement with earlier observations of Pachence et al. (1979) on lecithin–RCP proteoliposomes. These

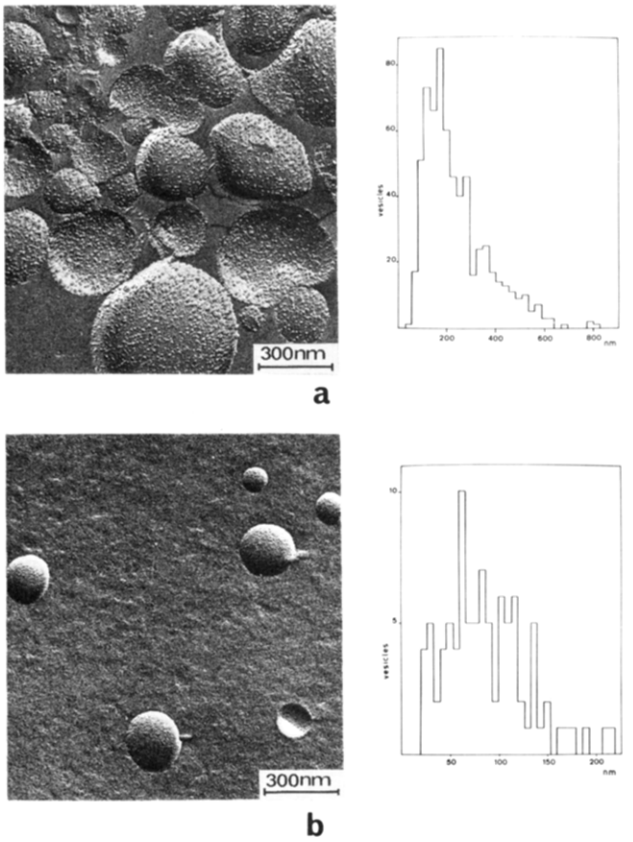

FIGURE 2: Freeze–fracture electron micrographs and histograms of vesicle diameters of (a) reconstituted proteoliposomes with a protein to lipid ratio of 0.57 (w/w). The histogram was determined on 635 vesicles (top). (b) Large liposomes used for the neutron scattering experiments; histogram measured on 93 vesicles (bottom).

changes were more clearly demonstrated by difference spectra taken between proteoliposomes before and after disruption by detergent (Figure 3). They showed an enhancement and broadening of the 869-nm band, especially on its far-infrared side, and a slight red shift of the 805-nm band; a decrease of the bacteriopheophytin band was also observed. A "flattening

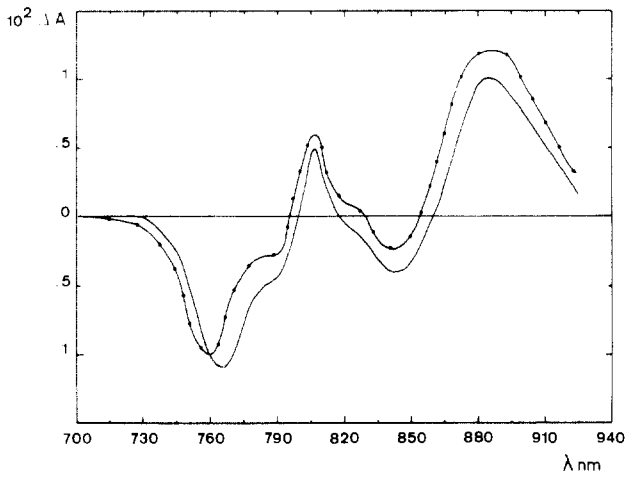

FIGURE 3: Difference spectrum of RCP in proteoliposomes vs. RCP in detergent solution. A base line was drawn between two split tandem cuvettes filled each with proteoliposomes (2.5 μM RCP) in the first chamber and buffer in the second. Then, 20 μL of buffer was added to proteoliposome in the sample cuvette; in the reference, 20 μL of 10% Triton X-100 was added to the proteoliposomes, and buffer was replaced by pure liposomes in the second chamber, adjusting their concentration to equilibrate the turbidities of the sample and the reference as to give ΔA (1000 nm) = 0. A difference spectrum was then recorded and corrected from the base line. The two difference spectra were obtained in independent experiments.

effect" (Duysens, 1956) could explain the later change but not that of the Bchl bands, which might be due to the nature of the environment (lipid or LDAO).

A general question concerning reconstitution is the asymmetry that results. The above-described evidence is in favor of the protein molecules being all incorporated into the lipid vesicles in the same manner, due probably to vectorial insertion of the RCP from the water phase into preformed liposomes. This asymmetry of the proteoliposomes is, however, not revealed on freeze–fracture images (Figure 2). Such freeze–fracture behavior may indicate either that the proteoliposomes are built up from symmetrically anchored proteins or that the protein molecules are almost equally anchored in the solvent on both sides of the proteoliposomes, leading to the equal probability for a given protein to remain either on the inner or on the outer half of the fractured vesicles. The latter interpretation is in a better agreement with the totality of results presented here and particularly with the model derived from the neutron diffusion data presented below. An asymmetric distribution of RCP was also observed for addition of RCP to lecithin liposomes followed by removal of LDAO (Pachence et al., 1979). A more random orientation was observed with the lecithin/RCP/cholate method (Overfield & Wraight, 1980a), where dissolution is homogeneous in lipid–detergent mixed micelles. Differences have also been observed according to whether the lipid is phosphatidylcholine or phosphatidylserine (Overfield & Wraight, 1980b), presumably as a result of electrostatic effects. Although information on the asymmetry of insertion is to some extent limited, similar types of experiments have been performed on bacteriorhodopsin, which also shows effects of the method of reconstitution (Hwang & Stoeckenius, 1977).

Characterization of Liposomes. For the neutron scattering experiments, large unilamellar vesicles of pure lecithin were used as a control (see below). Figure 1B shows chromatographic results for liposomes produced by reverse evaporation; a broad distribution of sizes is evident. For the neutron experiments, a pooled and concentrated fraction consisting of the main peak of elution was used. Its freeze–fracture images showed unilamellar vesicles, with a mean diameter of 1200 ± 600 Å (Figure 2b).

Neutron Scattering Results. Four preparations were used with protein volume fractions (C_{vp}) of 0, 0.32, 0.27, and 0.22. Figure 4a shows an example for the fourth (C_{vp} = 0.22). This preparation gave the least linear plot as the result of a "ripple" structure at low s^2, which is attributable to membrane curvature. The ripples are much less well defined in comparison with chromatophores [see Sadler & Worcester (1982)] and are absent for the samples with C_{vp} = 0 and 0.32. The position of the broad and shallow shoulder (s = 0.0043 $Å^{-1}$) gives a diameter of 360 Å with the same treatment as Sadler & Worcester (1982), which corresponds to the low end of the wide distribution of diameters seen by microscopy. The majority of the vesicles, since they are so large, would give no structure in this plot. Figure 4b shows an equivalent result for the C_{vp} = 0.22 preparation at relatively low ^2H_2O content (0.45 of ^2H_2O). Any ripple is even less apparent, owing to the increase in the statistical errors in the measurements.

A series of measurements were made at ^2H_2O fractions near ρ = 0. Over a range of ^2H_2O fraction of about 0.05, the intensity does not vary with ^2H_2O fraction. This intensity was small (Figure 4c) but reproducible. Equation 10 was used in order to test whether an intensity measurement of this type was consistent with the formalism. The value of ρ_l was obtained by using the measured value of α (see below) and eq 8.

The solid curve in Figure 5 shows the resulting calculated intensity for a homogeneous preparation. If a range of $\bar{\rho}$ values is used, the broken curves result. The scaling of the intensity was so as to give agreement with the high $\bar{\rho}$ case (Figure 4a). It can be seen first that intensities of comparable magnitude to those in Figure 4c can be generated and second that some heterogeneity (corresponding to about 0.06 in ^2H_2O content) is likely. This is not unreasonable compared with the characterization where the very approximate estimate of the rms differences in protein to lipid ratios correspond to differences of about 0.02 in ^2H_2O content. No attempt was made to pursue the fits of the "match-point intensities" further: neither the density profile nor the knowledge of the heterogeneity is adequate. The analysis is sufficient for the purpose of showing the absence of anomalous signals.

Figure 4d shows results at high $\bar{\rho}$ values for lipid vesicles. Analogous plots have been given previously for lipids (Knoll et al., 1981a,b), mainly on multilayered liposomes and (small) sonicated vesicles.

The analysis can now proceed by using eq 2 and 3. The intercepts from eq 2 were plotted as $I^1(s)s^2$ ($s \rightarrow 0$) against ^2H_2O fractional content. Figure 6a shows the result for the third proteoliposome preparation (C_{vp} = 0.22) and Figure 6b for lipid vesicles. The minima were interpreted as a change in sign of $\bar{\rho}$, in the usual way (eq 2). The average scattering length density of the membranes (the intercept) and the slope were obtained by weighted least-squares fits. From the slope, the quantity t can be derived. Table I includes the results. Two of the preparations give good agreement with the values of t expected on the basis of the D_w values (somewhat above 40 Å, see below). Two preparations, however, give rather low values of t (these were measured on the same occasion). The significance of these agreements and disagreements is not clear, since these are probably systematic errors both in the measurements of concentrations of suspensions and in the calibrations of the neutron scattering intensities.

The value of ^2H_2O fraction for $\bar{\rho}$ = 0 is entered in Table I and there is very good agreement with the known chemical

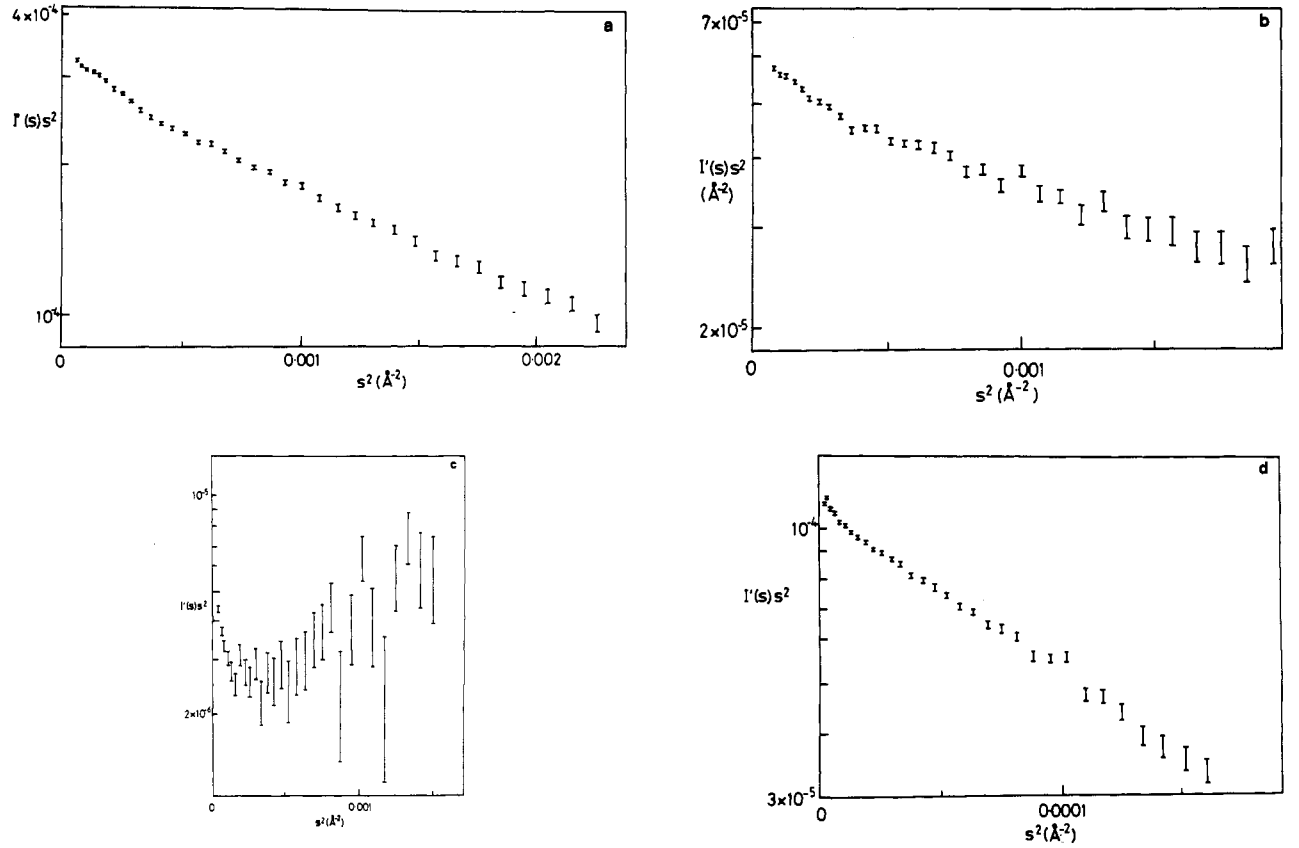

FIGURE 4: Guinier plots for sheets of $I^1(s)s^2$ on a logarithmic scale vs. s^2. The methods of data reduction are described in the text. The units of intensity are count rate divided by the sample transmission and by the count rate for ^1H_2O. (a–c) Proteoliposomes (RCP reconstituted with lecithin, with volume fraction of protein 0.22) with ^2H_2O fractions of 0.8, 0.45, and 0.17, respectively. (d) Lipid vesicles. See also Table I.

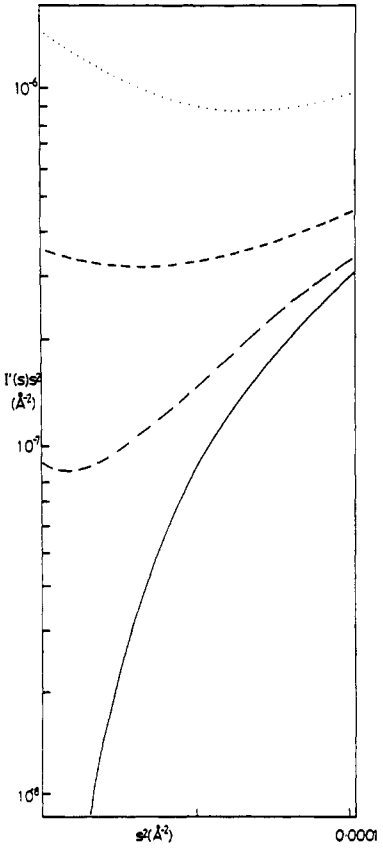

FIGURE 5: Curves of $I^1(s)s^2$ calculated for a two-phase model from eq 8, on a scale so as to compare with Figure 4d. The lowest curve corresponds to no heterogeneity, the other corresponds to heterogeneities obeying Gaussian distribution with standard deviations (in ascending order) of 0.02, 0.04, and 0.06 in ^2H_2O fraction.

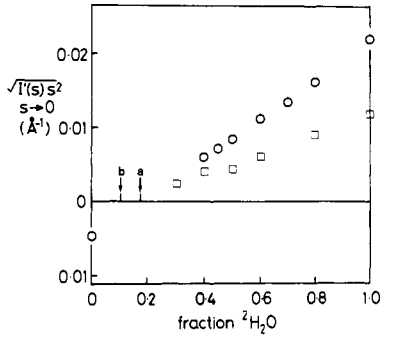

FIGURE 6: Square roots of intensity intercepts (from eq 5) plotted vs. ^2H_2O fraction. The statistical precision of the points according to the least-square fits is less than the size of the symbols. More important errors are probably due to uncertainties in concentrations. (a) Proteoliposomes (RCP protein volume fraction 0.22) (○); (b) lipid vesicles (□). Arrows indicate the intercepts. Results of least-squares fits to these plots are shown in Table I: average membrane scattering length densities (from intercepts) and t values (from slopes).

constitutions. Calculated match points are also included, on the basis of scattering length densities given by Sadler & Worcester (1982).

Figure 7 shows plots according to eq 3 for (a) proteoliposomes and (b) lipids. The plots cannot be used to derive a β value. For example, if a quadratic fit is used for proteoliposomes (C_{pv} = 0.22), a typical value for β is (8 ± 40) × 10^{-12} Å^{-2}. The main experimental restriction is the difficulty of obtaining data for positive $\bar{\rho}$ since the match points are at such low ^2H_2O content. In addition, the fact that the signals decrease so fast with s means that slight systematic background subtraction errors (fairly independent of s) lead to substantial changes in slopes and, hence, in D values. For lipids, the concentration was the lowest, and no measurement at all was

Table I: Summary of the Neutron Scattering Results[a]

	vol concn of suspension	C_{vp}	^2H_2O fraction for $\bar{\rho} = 0$	^2H_2O fraction for $\bar{\rho} = 0$ (predicted)	t (Å)	λ (Å)	instrument	D_w	$\alpha \times 10^4$	l (Å)	L (Å)
lipid	0.045	0	0.116 ± 0.026	0.118	27	10	D17[b]	12.5 ± 0.4	0.9 ± 0.4	43.3	
lipid + RCP	0.092	0.316	0.195 ± 0.005	0.20	42.9	6	D11	13.6 ± 0.5	1.6 ± 0.2	41.7	57
lipid + RCP	0.159	0.273	0.162 ± 0.004	0.19	41.3	11	D17	13.2 ± 0.2	0.9 ± 0.2	41.7	54
lipid + RCP	0.118	0.22	0.17 ± 0.008	0.18	33.3	10	D17[b]	12.8 ± 0.2	1.1 ± 0.2	41.7	52

[a] Four preparations have been investigated. The C_{vp} values (volume fraction) were calculated from the biochemical analysis (concentrations in milligrams per milliliter) and protein and lipid densities of 1.32 and 1.0 g cm^{-3}, respectively. The probable error in C_{vp} as judged by the reproducibility is at least as good as 5%. The uncertainties when they are shown are derived from the least-squares fit. The uncertainty in the L values depends to some extent on the choice of D_w and α for the pure lipid (see text). The most probable value is about 53 Å compared with an l value of 42 Å. It would be reasonable to give an uncertainty of no more than 2 Å for the purpose of comparing L and l values, though there are probably additional systematic effects. [b] The same 24-h experiment.

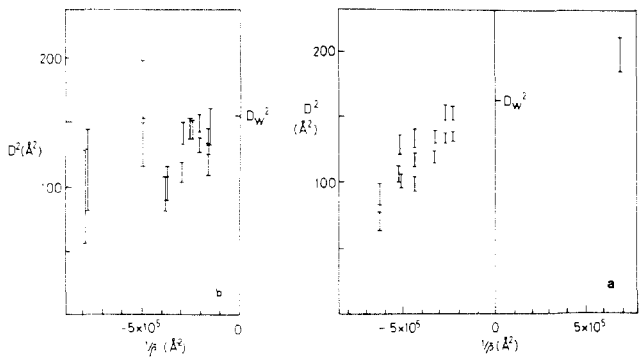

FIGURE 7: Plots of D^2 values (from plots as in Figure 4) against inverse contrast $\bar{\rho}$ (as derived from plots in Figure 6), according to eq 2. (a) RCP volume fraction 0.22; because of the small but systematic distortion in the Guinier plots (Figure 4a–c), different fits at the same contrast differ by more than the statistical precision. The distortion can be attributed to membrane curvature. Only a small number of intensity points contributed to the result at positive contrast. (b) Lipids. Results of straight line least-squares fits (variance weighted) are shown in Table I, giving D_w (from the intercepts) and α (from the slopes).

feasible at positive $\bar{\rho}$. Values for D_w and α from linear least-square fits are shown in Table I. As discussed below, however, the certainty in D_w values for lipids is better than implied by least-square fits since independent information is available concerning α.

Interpretation of Scattering Results. The simplest system for comparisons with expectations is the pure lecithin vesicles. The "dry" thickness of such layers is 40 Å (Reiss-Husson, 1967; Luzzatti, 1968) as described from lamellar periodicities and known water content in stacks of lipid layers. If the D_w value for lipids is interpreted in terms of layers with strictly planar surfaces, the thickness would be 43.3 Å. The difference between these thickness values can be interpreted in terms of irregularity in the surfaces and hydration of the head groups: both these effects would lead to a trapezoidal form for the density profile $\rho_w(z)$. The precision in the α measurement (Table I) is too low for the experimental value of α to be useful. The precision in D^2 values is relatively good at high ^2H_2O content, and the uncertainty in D_w can be attributed to the large uncertainties in D^2 values at low $\bar{\rho}$, which in turn leads to the uncertain slope. Hence, the uncertainties in the slope (α value) and in D_w are linked, and to assess that of D_w, it is useful to consider predicted values of α. The lipid layers are chemically simple and have been thoroughly characterized by methods including X-ray crystal diffraction (Pearson & Pascher, 1979).

Calculations based on homogeneous layers for head groups and hydrocarbon tails [see Sadler & Worcester (1982)] would give an α value of 1.08×10^{-4}. This is too high since the head group is not uniform in scattering length density: the $N(CH_3)_3$ group has the lowest density of the head group and yet has the highest z values. Hence, it will give a negative contribution to α. With the crystal structure (Pearson & Pascher, 1979) taken as the starting point, α was calculated from a sum over all atoms according to eq 8 as 0.51×10^{-4} or 0.44×10^{-4}, depending on whether the choline and phosphate groups are kept as they are in the crystals or whether they are extended outward to the maximum extent. The actual structure is presumably intermediate between these extremes (Büldt & Wohlgemuth, 1981). The calculations were performed by taking the conformation in the lipid crystal and tilting the molecule as a whole so as to give reasonable values for D_w for the monolayer state with fluid hydrocarbon, as in the vesicles. They show clearly that large discrepancies occur on assumption that the density distributions $\rho(z)$ are simple step functions: in this case, an overestimate of α by about a factor of 2. Of course, this effect is most pronounced for systems such as lipid layers giving small distance parameters ($D \simeq 10$ Å); for larger distance parameters, the neglect of detailed molecular structure will be less important. Even if some of the basic postulates of the contrast variation method did not hold strictly, it would be difficult to explain an α value very different from 0.5×10^{-4}. Preferential association of protons or deuterons at the head group would have to be very substantial (clearly, more than would be involved with only one or two water molecules), and there is no precedence for such an effect. Bound water effects have very little influence on $\rho_F(z)$ since match point for lipids is near zero scattering length density. If the expected value of α, 0.5×10^{-4}, is imposed on the data, a D_w value would be obtained that is lower than 12.5 Å and nearer the "minimum" value of 12 Å. Recent experiments that make use of deuterated lipids (to be published) confirm that D_w is indeed near the low end of the range, consistent with the present experiments.

For membranes with proteins, calculations in terms of molecular structure are less detailed. It is already clear that approximating proteins as regions of single scattering length density (ρ) is not adequate even for these low-angle experiments. Matters are improved by using two values for ρ for proteins (corresponding to hydrophilic and hydrophobic residues), but detailed models for the density fraction $\rho_F(z)$ are still not feasible (Sadler & Worcester, 1982). However, the formalism of contrast variation gives information in the form of D_w concerning $\rho_w(z)$, for which there are no such difficulties.

Calculations can conveniently be related to simple models where proteins project from the membrane surfaces as cylinders rectangular in cross section and of length L, l being the width of the lipid bilayer

$$D_w^2 = \frac{1}{12}[L^3W_p + l^3(1 - W_p)]/[LW_p + l(1 - W_p)]$$

where W_p in the fractional area of the membrane occupied by the protein. The volume fraction of protein C_{vp} is then

$$C_{vp} = W_pL/[W_pL + (1 - W_p)l]$$

In this way, D_w values lead to a length L for the cylinder and, hence, to an idea of how far the protein projects. Other shapes for the projecting parts of the proteins could of course be chosen (Sadler & Worcester, 1982). For intact photosynthetic membranes, L was 54 Å. L values are calculated for the RCP (Table I) in a similar way. Since C_{vp} values are so much lower than those for intact membranes, the reliability of a thickness value for the lipid is more important in this case. A 1% error in ρ_w for proteoliposomes leads to an error of approximately 0.6% in L; a 1% error in l leads to 0.4%. Although C_{vp} values are small, D_w for the proteoliposomes is still more important than l in determining L. Both because of the argument concerning α values that are expected for lipids (see above) and because of more precise recent measurements, we choose an l value corresponding to a lipid D_W value of 12 Å. L values are shown in Table I on this basis. L values decrease by 10% as C_{vp} is decreased, which is a rather bigger effect than would be expected on the basis of the precision in the measurements. This apparent discrepancy may be because the assumption of constant l is not valid, in which case the lipid thickness is increased by the presence of protein. If this is the case, then the correct L value would be even less than that in Table I.

Values of α do not lead to any detailed model, as mentioned above. However, it is significant that α is not markedly lower for proteoliposomes than it is for lipids. A decrease would be expected if ρ were to be uniform over the protein. As before (Sadler & Worcester, 1982), it can be inferred from the sign of α that the protein residues with lower ρ values (in general, the hydrophobic ones) and, possibly, the pigments tend to occur at the membrane center rather than the regions toward the surfaces.

From eq 9 it is clear that β depends only on $\rho_F(z)$. In other words, if $\rho_F(z)$ is zero or small, β is very small whatever the asymmetry in (say) the way the protein projects more on one side of the membrane than on the other. Although the precision of any measure of β is too poor for any definite conclusion, Figure 7a does hint that with more measurements at positive $\bar{\rho}$ it might be possible to measure a finite β. It should be emphasized that these measurements are not inconsistent with a large degree of asymmetry. For example, if all the hydrophilic residues were to be on one side for this type of membrane, β would only be 5×10^{-11} Å (Sadler & Worcester, 1982), i.e., still within the experimental error in β.

Discussion of the Protein Structure. The particular characteristics of these types of measurement are (a) contrast variation techniques enable results to be obtained independently of the fluctuations in ρ within the membrane, (b) thickness values are very sensitive to material at high z (projections from the membrane), and (c) the precision of the analysis is summarized in a simple and direct way from the fits to plots such as in Figure 7. For a favorable system, a set of measurements can be made in a few hours on the instruments D11 or D17 (at least, two sets of data per day).

The need to compare results for proteoliposomes and lipids has introduced some uncertainty, but the preceding section has shown that a value for the length of the RCP can be nevertheless derived. It is interesting to compare this result with those using diffraction from multilayers (Pachence et al., 1979, 1981, 1983), which use scattering data to much higher s values. A model from this work shows a protein placed asymmetrically in the membrane with a total length of about 57 Å with proteins from different membranes either in contact or separated by 3 Å, depending on whether the membrane faces are external or internal. The higher "resolution" as a result of using higher s values no doubt helps in deriving the details of structure within the protein and the asymmetry, but for a sensitive test of the length of the RCP, the suspension scattering technique is probably preferable. In addition to the type of diffraction technique used, there is also the important difference in hydration: in the stacks, the membranes were partially dehydrated. As a consequence, as is well-known from studies on lipid layers (Reis-Husson, 1967; Luzzati, 1968), the lipid layer is thicker (51 Å) than expected for the fully hydrated state (40 Å). It is not unconceivable that changing the environment of the protein could slightly distort (elongate) its conformation, though in both studies the spectral properties indicate a basically "native" conformation. In spite of L being slightly smaller, the ratio L/l is slightly greater (1.26) in the present study than can be derived (1.12) from the previous work (Pachence et al., 1979). Hence, the principal feature is the same: relatively small projections of the protein beyond the lipid layer. In addition, there are suggestions that the geometries of the protein and lipid may be interdependent in the following sense. The different results for L in Table I are consistent with a thickening of the lipid as a result of the presence of protein. If our results are compared with previous diffraction results, better consistency is achieved on the basis that thickening the lipid layer by partial drying may influence the protein.

Measurements on intact photosynthetic membranes gave an average protein "length" L of 54 Å, which is small compared with general expectations (at least as expressed in schematic diagrams of membranes). One reason for this could have been the proteins associated with the light-harvesting chlorophyll, which are of such low molecular weight (Cogdell & Thornber, 1980) that they would not be able to project far from the lipid layer. This now seems unlikely to be the only explanation, since the RCP has an even smaller L value despite its substantial molecular weight (84 000). The only detailed structure determination for a hydrophobic membrane (Henderson & Unwin, 1979) has shown a compact structure with the protein embedded almost entirely within a depth comparable with the lipid layer. The measurements on proteoliposomes suggest that this is also true for the RCP. Measurements of infrared dichroism, related to the orientation of α helices, also show similarities between the purple membranes (Rothschild & Clark, 1979) and the photosynthetic membrane (Nabedryk & Breton, 1981; Nabedryk et al., 1982).

There is a significant preference for molecular groups in the RCP of high ρ values (hydrophilic) to be preferentially near the membrane surfaces.

Acknowledgments

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A Rapid Method of Reconstituting Human Erythrocyte Sugar Transport Proteins†

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ABSTRACT: A rapid reconstitution procedure for human erythrocyte hexose transfer activity is described. The procedure (reverse-phase evaporation) avoids exposure of the isolated proteins to detergent, organic solvent, sonication, or freeze–thaw steps during insertion into synthetic membranes and may be effected within 15 min. The so-formed vesicles are unilamellar structures with a large encapsulated volume, narrow size range, and low passive permeabilities. Contamination by carry-through of endogenous (red cell) lipids is less than 1%. Reconstituted hexose transfer activity was examined by using unfractionated proteins (bands 3, 4.5, and 6) and purified proteins (bands 4.5 and 3). With unfractionated proteins, hexose transport activity is low [0.34 μmol·(mg of protein)$^{-1}$·min^{-1}], is inhibited by cytochalasin B, and increases monotonically with protein concentration. Kinetic analysis indicates that V_{max} values for both influx and efflux of D-glucose are identical. Reconstitution of the cytochalasin B binding protein (band 4.5) results in hexose transport with high specific activity [5 μmol·(mg of protein)$^{-1}$·min^{-1}] and symmetry in transfer kinetics. Band 3 proteins also appear to mediate cytochalasin B sensitive D-glucose transport activity.

The sugar transport proteins of the human erythrocyte membrane have been reconstituted into synthetic membranes by a variety of means. These methods include reconstitution by detergent dialysis (Goldin & Rhoden, 1978), reconstitution by insertion into planar black lipid membranes (Jones & Nickson, 1978), or reconstitution by a freeze–thaw, sonication procedure (Kasahara & Hinkle, 1977; Wheeler & Hinkle, 1981). Here we report a rapid reconstitution procedure that obviates the need for exposing proteins to detergent, organic solvent, sonication, or freeze–thaw cycles during insertion into synthetic membranes (reverse-phase evaporation; Szoka & Paphadjopoulos, 1980; Düzgünes et al., 1983). The vesicles formed by this procedure are large unilamellar structures of a narrow size range, with hexose transfer characteristics similar

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