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An X-ray diffraction analysis of oriented lipid multilayers containing basic proteins

W. MacNaughtan[a,*], K.A. Snook[a], E. Caspi[b] and N.P. Franks[a,**]

[a] *Biophysics Section, Blackett Laboratory, Imperial College of Science & Technology, Prince Consort Road, London, SW7 2BZ (U.K.) and* [b] *Department of Bio-Organic Chemistry, The Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545 (U.S.A.)*

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X-ray diffraction techniques have been used to study the structures of lipid bilayers containing basic proteins. Highly ordered multilayer specimens have been formed by using the Langmuir-Blodgett method in which a solid support is passed through a lipid monolayer held at constant surface pressure at an air/water interface. If the lipid monolayer contains acidic lipids then basic proteins in the aqueous subphase are transferred with the monolayer and incorporated into the multi-membrane stack. X-ray diffraction patterns have been recorded from multilayers of cerebroside sulphate and 40% (molar) cholesterol both with and without polylysine, cytochrome *c* and the basic protein from central nervous system myelin. Electron density profiles across the membranes have been derived at between 6 Å and 12 Å resolution. All of the membrane profiles have been placed on an absolute scale of electron density by the isomorphous exchange of cholesterol with a brominated cholesterol analog. The distributions and conformations of the various basic proteins incorporated within the cerebroside sulphate/cholesterol bilayer are very different. Polylysine attaches to the surface of the lipid bilayer as a fully extended chain while cytochrome *c* maintains its native structure and attaches to the bilayer surface with its short axis approximately perpendicular to the membrane plane. The myelin basic protein associates intimately with the lipid headgroups in the form of an extended molecule, yet its dimension perpendicular to the plane of the membrane of approx. 15 Å is consistent with the considerable degree of secondary structure found in solution. In the membrane plane, the myelin basic protein extends to cover an area of about 2500 Å^2. There is no significant penetration of the protein into the hydrocarbon region of the bilayer or, indeed, beyond the position of the sulphate group of the cerebroside sulphate molecule.

Introduction

The basic protein from central nervous system myelin membranes is one of the most extensively studied membrane proteins. Although it is now more than two decades since it was first isolated [1], both its interactions within the myelin sheath and its physiological role remain obscure. The myelin basic protein constitutes about 30% (by weight) of the total protein in central nerve myelin. It is a relatively small protein with a molecular weight of about 18500, and it has an unusual primary sequence which is rich in basic residues. Its high net positive charge at physiological pH means that it could interact strongly with the headgroups of acidic lipids, but its relative ease of extraction from the intact myelin membrane clas-

* Present address: Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

** To whom all correspondence should be addressed.

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sifies it as essentially extrinsic to the myelin lipid bilayer.

There have been many studies on the interactions between the myelin basic protein and lipids, and it is well established that the predominant forces which are acting are electrostatic in nature. There is, however, a considerable body of evidence which has been interpreted as showing that the protein penetrates to some degree into the hydrocarbon region of the lipid bilayer, where hydrophobic segments of the protein are said to interact with the lipid hydrocarbon chains (for reviews, see Refs. 2–6). Most of this evidence is, however, rather indirect, with protein penetration being inferred from observed changes in, for example, membrane permeability [7,8], lipid phase transition temperature [8,9] or monolayer surface pressure [10].

There have been a few attempts to provide more direct information about the conformation and distribution of the myelin basic protein by using X-ray diffraction techniques to study the structures of membranes containing the protein [11–13]. These studies, however, were hindered by the low resolution of the diffraction data and by the difficulty of obtaining a common scale for the membrane electron density profiles.

The aim of the present work was to provide more definitive information on the distribution of the myelin basic protein within a lipid bilayer, including the degree to which it penetrates into the bilayer, and to compare its conformation and distribution with those of other basic proteins. We hoped to circumvent the difficulties encountered in previous work by obtaining much higher resolution diffraction patterns and by placing all our derived electron density profiles on an absolute scale. We therefore developed a novel method [14] of specimen preparation based on the Langmuir-Blodgett technique which provided homogeneous and highly ordered multi-membrane stacks containing either the myelin basic protein, cytochrome *c* or polylysine. The diffraction patterns from these specimens have been analysed to give relatively high resolution electron density profiles, which have been placed on an absolute scale of electron density using a method based on the isomorphous exchange of halogenated cholesterol [15]. We chose to study in detail the interactions between the basic proteins and a lipid bilayer system which contained a myelin lipid (cerebroside sulphate) known to interact particularly strongly with the myelin basic protein in vitro [10,16,17]. Our lipid bilayer, like that of the intact myelin membrane, also contained 40% molar cholesterol, so the hydrocarbon region was maintained in a fluid liquid-crystalline state. Our diffraction analysis should provide a framework for interpreting the results obtained using similar lipid bilayer systems and other spectroscopic techniques.

Materials and Methods

Materials. Myelin basic protein (rabbit brain) was obtained from Calbiochem-Behring and cerebroside sulphate (sulphatides, bovine) was obtained from Supelco. Cytochrome *c* (horse heart, type VI), cholesterol (chromatography grade), phosphatidylserine (bovine brain), poly (L-lysine) (average molecular weight 17000 $g \cdot mol^{-1}$), poly (L-arginine) (average molecular weight 44000 $g \cdot mol^{-1}$), L-lysine and L-arginine were obtained from Sigma. [26,27-^{14}C]Cholesterol was obtained from Amersham International, and the spinal cord lipids were prepared by Lipid Products Ltd.

Langmuir-Blodgett apparatus. The Langmuir-Blodgett trough was machined from a single piece of PTFE and had internal dimensions of approximately $28 \times 20 \times 2$ cm^3. The trough was bolted to a dural plate (thickness = 2.5 cm) which was supported on a kinematic anti-vibration mount. The lipid monolayer was compressed using a PTFE barrier, operated by a stepping motor. The barrier was driven along a precision lead-screw (1 mm pitch) on a carrier specially designed to operate smoothly in the absence of lubricant with the absolute minimum of backlash. The surface pressure of the monolayer was monitored using a Wilhelmy plate (Whatman No. 1 filter paper [18]), supported by a sensitive force transducer. The signal from the force transducer was fed to electronics which controlled the stepping motor. The surface pressure of the monolayer could be held constant to within ± 0.1 $mN \cdot m^{-1}$. For the preparation of multilayers (see next section) a solid substrate was moved vertically through the monolayer at a constant speed (using a heart-shaped cam driven by a servo motor). The speed of the

substrate was continuously variable between 0.06 and 15 $mm \cdot s^{-1}$ and the speeds for the up and down strokes could be set independently. The entire apparatus was used in a laminar-flow bench, which was sited in a temperature-controlled room (held at $18 \pm 1°C$). For further details see reference [19].

Preparation of multilayer specimens. All of the multilayer specimens which contained protein were prepared using the Langmuir-Blodgett technique. The basic principle of the method is to pass a solid substrate through a monolayer held at constant surface pressure at an air/water interface. Under the appropriate conditions the monolayer will be transferred to the solid substrate either during the down stroke, the up stroke, or both. If protein molecules are present in the aqueous subphase beneath the lipid monolayer, and if they interact sufficiently strongly with it, then protein will be transferred with the monolayer and incorporated into the multilayer stack [14]. The concentration of protein in the aqueous subphase ranged from 130 to 330 nM. At these low protein concentrations, we observed no significant surface-active contamination over a period very much longer than that taken to lay and compress the lipid monolayer. Experiments were performed at either pH 7.0 (10 mM Tris acetate) or pH 5.5 (glass-distilled water passed through a milli-Q system) with essentially identical results, although in the presence of low salt concentrations (10 mM), somewhat less protein was transferred with the monolayer. At high concentrations (100 mM) of either Tris acetate or Hepes buffer in the aqueous subphase, we obtained little or no monolayer transfer to the substrate. Most experiments were performed using pure water. The lipid monolayer was formed in the conventional way, using a few drops of a solution of the lipids in either chloroform or chloroform/methanol (10 : 1, v/v). The solvent was allowed to evaporate and the lipid monolayer was then slowly compressed to a chosen surface pressure. Multilayer specimens were usually made when the monolayer was compressed to 40 $mN \cdot m^{-1}$. At this surface pressure the pressure/area isotherm was very steep and the packing density of the lipids was close to its limiting value; monolayer collapse occurred at about 50 $mN \cdot m^{-1}$.

The specimens were prepared on aluminium foil (0.075 mm thick, 1 cm square) which was curved (5 mm radius) to satisfy the requirements of the X-ray diffraction geometry [20]. The foil was cleaned by sonication in chloroform/methanol (1 : 1, v/v). During dipping, the foil was supported by a narrow neck, so that the geometrical area of the aluminium substrate could be accurately related to the area of the transferred monolayer. We found that, in order to obtain consistent and uniform monolayer transfer, the speeds of the up and down strokes were critical parameters. Trial and error showed that best results were obtained, under our conditions, when the substrate was moved fairly rapidly down through the monolayer (9.1 $mm \cdot s^{-1}$) and then withdrawn very slowly (0.08 $mm \cdot s^{-1}$). These speeds were used in all the experiments described here.

A record of the changes in monolayer area and pressure during a typical successful dipping cycle are shown in Fig. 1. During the fast downward

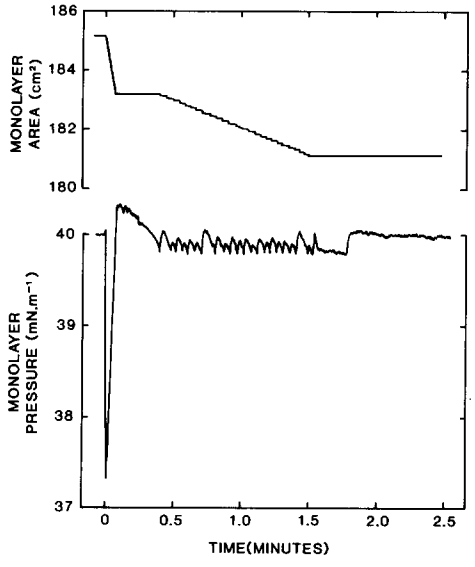

Fig. 1. Changes in monolayer area and pressure during the preparation of a Langmuir-Blodgett specimen. An aluminium substrate (area = 1 cm^2) is passed rapidly down through the monolayer (composed of cerebroside sulphate and 40% molar cholesterol) and withdrawn slowly. A monolayer is transferred to both sides of the substrate during each stroke; the changes in area and pressure shown are for a typical single dipping cycle after several monolayers have already been transferred. In this example the myelin basic protein is present in the aqueous subphase.

stroke the force transducer (approx. 15 cm away from the point of dipping) registers a sudden drop in pressure as the monolayer is rapidly transferred. The size of the decrease depends upon the total monolayer area and surface compressibility. The more rigid monolayers gave the larger pressure changes and the best transfer (as in Fig. 1), while the more fluid monolayers showed very small pressure changes and relatively poor transfer. For all monolayers, during the slow upward stroke, the monolayer pressure remained constant to within about ± 0.1 mN $\cdot$ m^{-1}. The area of the monolayer transferred during the cycle (see Fig. 1) divided by the geometrical area of the substrate defines the deposition ratio. For most samples this ratio (see. Fig. 2) was very close to unity, although transfer was slightly better during the slow upstroke compared with the fast downstroke. Multilayer specimens used for X-ray diffraction measurements usually consisted of between 30 and 50 bilayers, although excellent diffraction patterns could be recorded from as few as 20 bilayers (see also Ref. 21).

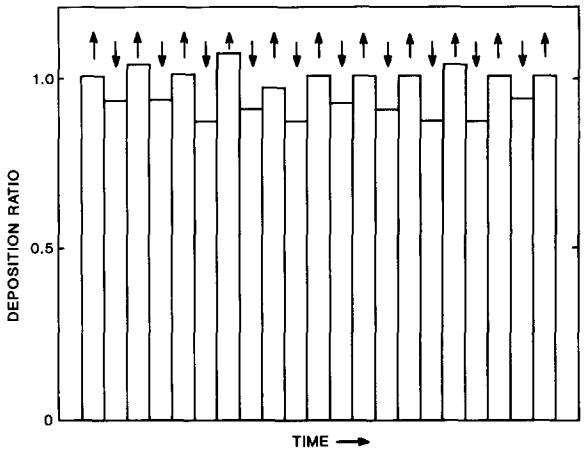

Fig. 2. The ratio between the area of the lipid monolayer transferred and the geometrical area of the aluminium substrate was close to unity. These data are for the preparation of a multi-membrane stack of cerebroside sulphate and 40% (molar) cholesterol membranes containing the myelin basic protein. The area of monolayer transferred during the fast downward stroke was always slightly less than the area transferred during the slow upward stroke.

If protein was not present in the aqueous subphase it was far more difficult, in general, to build up satisfactory multilayers (even in the presence of divalent cations such as calcium). Diffraction specimens of the lipids alone were therefore prepared using the solvent evaporation technique. Here a solution of the lipids in chloroform/methanol (10 : 1, v/v) was allowed to evaporate slowly on a curved glass support, under a gentle stream of moist nitrogen. A few specimens were also made by dispersing the lipid multilayer formed in this way in a 25 mM solution of calcium chloride; the lipids were then transferred to thin-walled glass capillary tubes which were centrifuged at about $500 \times g$ for 2 min before sealing.

X-ray diffraction methods. The X-ray diffraction measurements were made (at 23 ± 2°C) using single mirror and toroidal focussing cameras on Elliott GX18 and GX6 rotating anode generators. The multilayer samples were equilibrated with moist helium at various relative humidities in a diffraction chamber with aluminium windows (6 μm thick). The diffraction patterns were recorded on film (Reflex 25, CEA Verkan) or using a one-dimensional position sensitive detector (Marconi Avionics). Specimen-to-film distances were determined by recording the diffraction from calcite or molybdenum disulphide, which was sprinkled on the specimens at the end of the experiments. The X-ray films were densitometered on a Joyce-Loebl Scandig 3 microdensitometer using a 50 μm raster. The densitometer was controlled by a Data General Nova 3 computer using a densitometry program which integrated along the reflections (lines for the line focus geometry or arcs for the point-focussed geometry).

The corrected intensities for the lamellar reflections $I_{cor}(h)$ were obtained from the observed intensities $I_{obs}(h)$ (where h is the order number) using

$$I_{cor}(h) = I_{obs}(h) \frac{hA_f^{(n-1)/\cos(2\theta)}}{\frac{1}{2}(1+\cos^2 2\theta)}$$

where A_f is the film absorption factor (3.05 ± 0.15 for CEA film), n is the number of the film in the stack on which $I_{obs}(h)$ was measured and θ is the Bragg angle. Further details can be found in Refs. 20, 22 and 23.

Chemical analysis of the multilayer specimens. There were two principal problems in determining

accurate estimates of the protein and lipid content of our multilayer specimens: the first was the small quantity of material present in a typical sample of 50 membranes, and the second was the very strong interaction we found between the cerebroside sulphate and the basic proteins. This meant that we needed sensitive assays for both lipid and protein in which the presence of one component did not interfere with the assay of the other. We were finally successful in measuring the protein content of our specimens using a modified micro-Kjeldahl technique [24]. Briefly, the membranes were first removed from the aluminium substrate by sonication in a 1% solution of Triton X-100. Samples of this solution were then ashed using perchloric acid at 210°C, which converted all of the nitrogen in the sample to ammonia. The ammonia content was then measured using the indophenol blue method with ammonium sulphate as the standard. This technique proved to be amply sensitive and extremely reproducible and the results of a typical analysis are shown in Fig. 3. The single nitrogen in cerbroside sulphate contributed between 10 and 20% of the total nitrogen in the sample and was allowed for when calculating the protein content. By using known quantities of cytochrome *c* and selected amino acids we found that the protein determination was accurate to within $\pm 5\%$.

The lipid content of the multilayer specimens was determined using radioactively-labelled cholesterol. All of the multilayer specimens contained a very small percentage of [26,27-^{14}C]cholesterol. Small (100 μl) aliquots of the Triton X-100 solution which was used for the protein determination were added to 10 g of scintillation fluid (Beckman Ready-solv HP) and the activity measured using a Searle Isocap 300 scintillation counter. This activity was related to the mass of cholesterol by using known quantities of the [26,27-^{14}C]cholesterol as an internal standard.

Attempts to determine the water contents of our Langmuir-Blodgett multilayer samples proved unsuccessful. However, an accurate value for the water content of multilayers of pure lipids (prepared using the solvent evaporation technique) was obtained gravimetrically and found to be equivalent to 20 ± 1 water molecules per cerebroside sulphate molecule at 100% relative humidity.

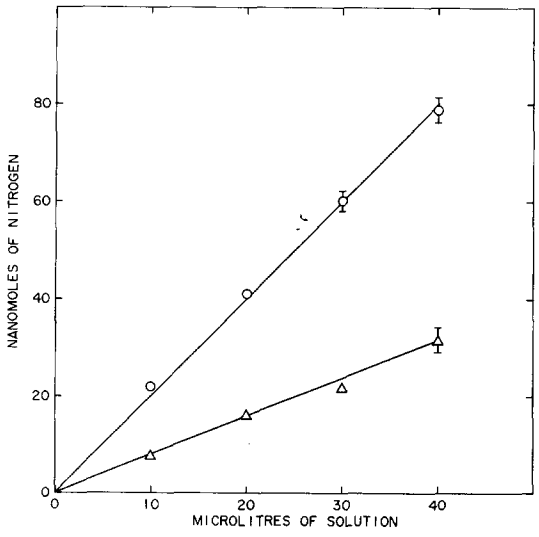

Fig. 3. The determination of the protein content of a multimembrane stack. The total nitrogen content in small aliquots of a 1 mM ammonium sulphate standard (○) and a Triton-X solution of 50 membranes of a cerebroside sulphate/cholesterol/myelin basic protein specimen (△). The slopes of the lines were obtained using least squares and the intercepts have been set to zero. The error bars represent the standard error in the mean. Where error bars are not shown they are less than the size of the symbol.

The mass densities of the lipids and proteins were determined with an Anton Paar precision density meter (DMA 602) using methods developed previously [25].

The determination of the absolute electron density scale. The high resolution electron density profile (see Results, Fig. 7) for the bilayer of lipids alone (cerebroside sulphate plus 40% molar cholesterol) provided the starting point for scaling the profiles. With the scale of this profile fixed, all other profiles for membranes containing normal cholesterol were scaled to it so as to minimize the differences over the hydrocarbon region (± 22 Å from the bilayer centre). Because of the well-defined levels of electron density at the bilayer centre and over the cholesterol nucleus region, this procedure gave unambiguous scale factors. The profiles for the bilayers containing either the myelin basic protein or cytochrome *c* were at significantly lower resolution than that for the lipids alone so before these profiles were scaled, an appropriate temperature factor was applied to the lipid data (see Results).

The profiles with normal and brominated

cholesterol were scaled together to minimize the differences over the headgroup regions [15]. The only difference between the normal and brominated cholesterol was that a single hydrogen atom on the terminal methyl group (C-26) had been replaced by a bromine atom. The areas under the difference profiles (Figs. 7, 11 and 13) were then used to calculate the multiplicative factor between the relative and absolute electron density scales [15]. The mean electron density of the lipid bilayer was obtained using our observed values of mass density (1.087 $g \cdot cm^{-3}$) and water content (20 ± 1 water molecules per cerebroside sulphate molecule when $D = 70.61$ Å) and a calculated value for the molecular weight [26] of cerebroside sulphate (877 $g \cdot mol^{-1}$) and found to be 0.354 electrons per $Å^3$. The corresponding cross-sectional area of the unit cell (one cerebroside sulphate molecule plus 2/3 cholesterol molecule) is 65.95 $Å^2$. This area is about 10% smaller than that found for total myelin lipids [27] and this difference may reflect either the degree of saturation of the hydrocarbon chains or the conformation of the polar headgroup.

In all the electron density profiles that we have derived, the lipid bilayer thickness, as defined by the distance between the sulphate groups, remained constant. To a good approximation, therefore, the cross-sectional area occupied by the lipid molecules also remained constant. On this basis we have placed our electron density profiles on an absolute scale. The good agreement between our directly measured protein contents and those derived from the difference electron density profiles (see Results), provides independent support for the accuracy of the absolute scale.

Results

The structure of bilayers of cerebroside sulphate and 40% molar cholesterol

Diffraction patterns were recorded from oriented lipid multilayers containing either normal cholesterol or the brominated cholesterol analog [15], over a range of relative humidities. Above 88% relative humidity all of the patterns looked qualitatively the same (see Figs. 4a and 4b). They consisted of a single series of 11 or 12 sharp lamellar reflections (out to a resolution of about 6 Å), together with a broad, equatorially-oriented diffraction band at about 4.5 Å showing the hydrocarbon region of the bilayer to be in a fluid liquid-crystalline state. Below 88% relative humidity there was evidence of increased disorder (the lamellar reflections broadened) and phase separation (additional reflections from crystalline cholesterol appeared). Consequently, only data recorded at and above 88% relative humidity has been used in the analysis.

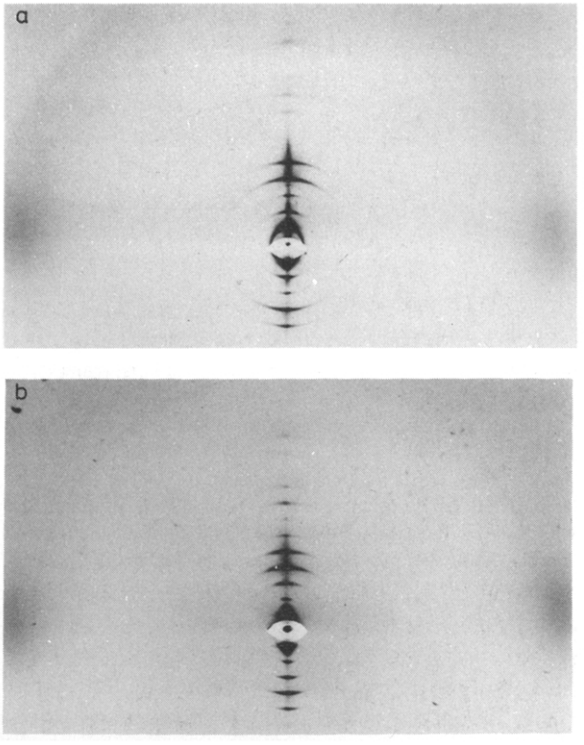

Fig. 4. Typical X-ray diffraction patterns from oriented multilayers of cerebroside sulphate and 40% (molar) cholesterol. (a) Normal cholesterol, $D = 70.6$ Å. (b) Brominated cholesterol, $D = 72.4$ Å.

Phases for the lamellar reflections were assigned using swelling methods [20] and a heavy atom method [15] based on the isomorphous exchange of the cholesterol analog. Both of the methods were applied to all of the structures described in this paper and enabled phases to be assigned with a fair degree of certainty. A typical swelling series is shown in Fig. 5. The structure factors are plotted together with a continuous Fourier transform, calculated using Shannon's theorem [28,29] and the data set at 100% relative humidity. All of the

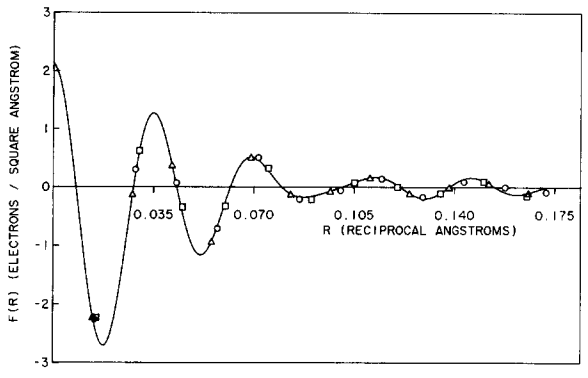

Fig. 5. A typical swelling series. X-ray structure factors are plotted vs. the reciprocal lattice vector R ($= 2 \sin \theta/\lambda$) for multilayers of cerebroside sulphate and 40% (molar) brominated cholesterol. The data points are for relative humidities of 100% (△), 97% (○) and 88% (□). The continuous Fourier transform has been calculated using Shannon's theorem with the data for 100% relative humidity. $F(0)$ was calculated from the known chemical composition and mass density of the bilayer (see text for details). Notice that all of the data points lie close to the calculated Fourier transform.

data points lie very close to the calculated transform. This is consistent with the basic assumption of the method–that the bilayer structure does not change greatly with swelling. This is also, of course, reflected in the derived electron density profiles, which are shown in Fig. 6. It can be seen that the changes that do occur are in the water spaces between the bilayer surfaces, as might reasonably be expected.

The electron density profiles for bilayers with

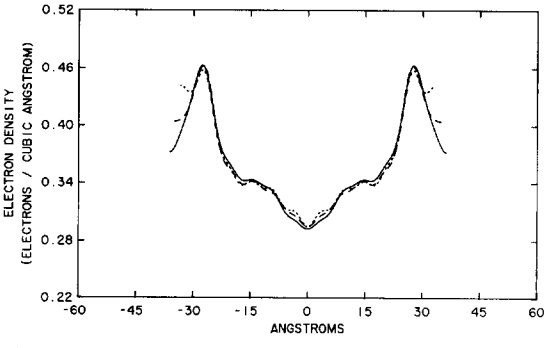

Fig. 6. Electron density profiles for cerebroside sulphate and 40% (molar) brominated cholesterol at three different relative humidities. The relative humidities are: 100% (solid line), 97% (dashed line), 88% (dotted line). The only significant changes in the electron density profiles occur in the water regions between the bilayer surfaces.

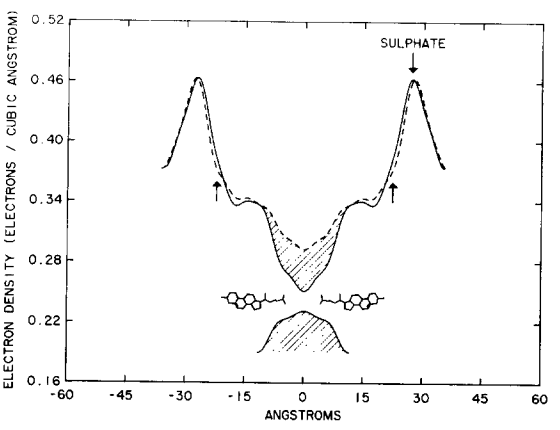

Fig. 7. Electron density profiles for bilayers of cerebroside sulphate with 40% (molar) normal cholesterol (solid line) and brominated cholesterol (dashed line) at 100% relative humidity. The difference profile shown below represents the distribution of bromine atoms and thus the positions of the terminal methyl groups of the cholesterol molecules. The arrows at ±22.2 Å indicate the calculated partial thickness of the hydrocarbon region in the bilayer (see text for details). The atomic co-ordinates for cholesterol (kindly supplied by Dr. B.M. Craven) are those from the three-dimensional co-ordinates of cholesterol in crystals of cholesterol monohydrate, projected onto a plane perpendicular to the long-axis of the molecule.

normal and brominated cholesterol are shown in Fig. 7. The difference between the two profiles shows the distribution of bromine atoms and thus reflects the positions of the terminal methyl groups of the cholesterol molecules. The major peaks of electron density at about ±27 Å from the bilayer centre can be interpreted as the positions of the sulphate groups of the cerebroside sulphate molecules. The broad peaks at about ±15 Å from the bilayer centre reflect the steroid nucleus of the cholesterol molecule [27] and position the molecule such that its hydroxyl group is about 21 Å from the bilayer centre. This can be compared to the partial thickness of the hydrocarbon region of ±22.2 Å (arrows on Fig. 7) which was calculated knowing the average chain-length and degree of saturation of the hydrocarbon chains of the cerebroside sulphate molecules [26] together with values for the mass densities of hydrocarbon and cholesterol [30,31].

In order to compare the bilayer structure in an oriented multilayer to that in excess water, we prepared multi-walled vesicles by dispersing the lipids in 25 mM calcium chloride. (In the absence of calcium the lipids do not form coherent multi-

walled structures.) The observed lamellar spacings were very similar to those measured for the oriented multilayers but the diffraction rings from the vesicles went out to much lower resolution (about 14.5 Å). Although at considerably lower resolution, the derived electron density profiles for the multi-walled vesicles showed the same features as the higher resolution structure with an almost identical headgroup-to-headgroup distance across the bilayer.

Meaningful comparisons between electron density profiles can only be made if the profiles are at comparable resolution [20,32]. However, the diffraction patterns that we recorded (see below) from protein-containing membranes were at a resolution which usually lay between that of the oriented lipid multilayers and that of the lipid vesicles. The procedure we adopted in order to compare the profiles for the protein-containing membranes to those for the lipids alone was to use only the higher resolution lipid data (from the oriented multilayers) but to compute the electron density profile at a resolution equal to that of the profile with which it was to be compared. This was done by first resampling the lipid data at the invariably larger lamellar spacing of the protein-containing membrane using Shannon's theorem [28,29]. Then a temperature factor was applied to the lipid data, so that the reflections beyond the resolution limit of the protein-containing system would not have been experimentally observable [20].

Preparation of multilayer specimens using the Langmuir-Blodgett technique

In virtually all cases it was possible to transfer three monolayers (see also Ref. 33) on to the solid substrate, but whether or not it was possible to build up further layers depended upon the nature and degree of the interaction between the protein and the lipid monolayer. For example, it was evident that some degree of non-covalent 'cross-linking' between the lipid headgroups (in the monolayer) by the protein molecules was a necessary requirement for good transfer. This is illustrated in Fig. 8, where the changes in the pressure and area of a monolayer of cerebroside sulphate and 40% molar cholesterol are plotted against time with either a basic polypeptide (Fig. 8a) or a basic amino acid monomer (Fig. 8c) in solution at similar mass concentrations beneath the lipid monolayer. The presence of the polypeptide resulted in excellent transfer of the monolayer, with the relatively large pressure spikes (pressure decreases) being consistent with transfer to the substrate of a fairly rigid monolayer. On the other hand, when only the amino acid monomer was in solution beneath the lipid monolayer, we could not build up more than three layers. After this, a monolayer was always shed back at the air/water interface during the fast downward

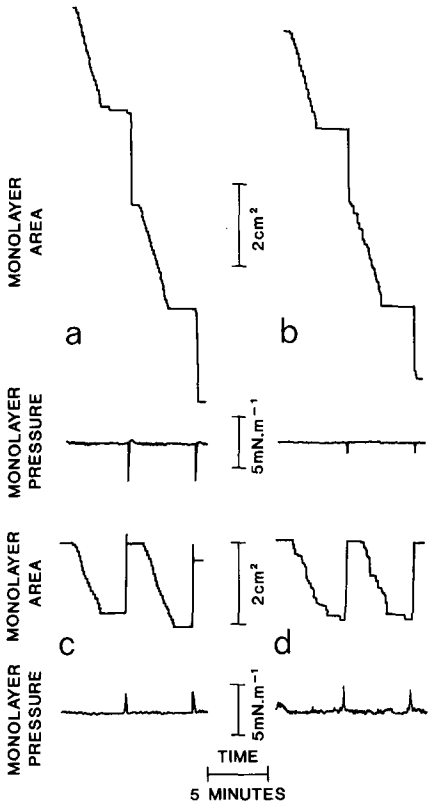

Fig. 8. A strong interaction between the protein and lipid is important for the successful formation of a multilayer stack. With cerebroside sulphate and 40% (molar) cholesterol a multilayer stack can be formed when a basic polypeptide such as polylysine or polyarginine is present in the aqueous subphase (Fig. 8a) but not in the presence of the amino acid monomer (Fig. 8c). Multilayers containing the myelin basic protein can be formed when the lipid monolayer contains a sufficient concentration of an acidic lipid, such as cerebroside sulphate and 40% molar cholesterol (Fig. 8b), but not when the monolayer contains no acidic lipids, such as dipalmitoyl phosphatidyl choline and 40% molar cholesterol (Fig. 8d).

stroke (accompanied by a sharp pressure increase), although it could be picked up again during the next slow upward stroke.

For a given protein, the ease of monolayer transfer depended greatly upon the composition of the lipid monolayer. With the myelin basic protein, for example, excellent multilayers could be made when the monolayer was composed of cerebroside sulphate and 40% molar cholesterol (Fig. 8b). However, as Fig. 8d shows, multilayer specimens could not be made when the cerebroside sulphate was replaced with dipalmitoylphosphatidylcholine (DPPC). With DPPC or cerebroside sulphate a monolayer was transferred during the slow upward stroke, but with DPPC, at the start of the next downward stroke, this was immediately shed at the air/water interface. For cerebroside sulphate the monolayer was transferred to the substrate during both up and down strokes so that a multilayer stack could be built up indefinitely. Several different lipids and lipid mixtures were tried. For most of these, quality and ease of monolayer transfer lay somewhere between the two extremes illustrated in Figs. 8a and d. For example, if the cerebroside sulphate concentration was reduced from 60% molar to 30% molar and replaced by 30% DPPC (both mixtures containing 40% molar cholesterol) then good transfer could be obtained at either 40 $mN \cdot m^{-1}$ or 30 $mN \cdot m^{-1}$. However, if the cerebroside sulphate content was further reduced to 22%, then transfer could only be obtained at the higher surface pressure. Multilayers could be made using phosphatidylserine and 40% molar cholesterol or with total spinal cord lipids, but in both cases the deposition ratio was variable and considerably less than unity. In addition, the diffraction patterns recorded from multilayers containing phosphatidylserine were poor and consistently showed the existence of more than one structure.

One further aspect concerning the interactions between the lipid monolayer and various proteins deserves comment. We consistently observed an increase with time in the surface pressure of the lipid monolayer when the aqueous subphase contained either the myelin basic protein or cytochrome *c*. This occurred at absolute surface pressures below about 25 $mN \cdot m^{-1}$ and reached equilibrium after about 10–15 minutes. Typically, the monolayer pressure started at about 10 $mN \cdot m^{-1}$ and increased to about 15 $mN \cdot m^{-1}$ during 5–10 minutes. In all other cases, with either the lipids alone or with basic polypeptides or amino acids in solution beneath the monolayer, we observed a pressure decrease with time.

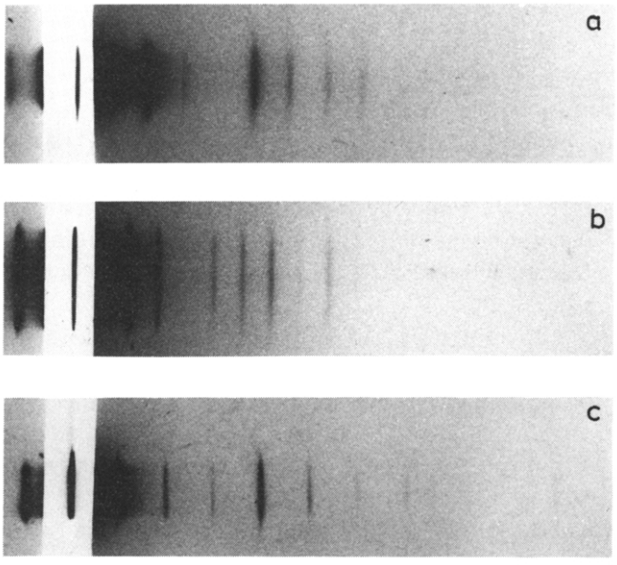

Fig. 9. Low-angle X-ray diffraction patterns of multilayers of lipids (cerebroside sulphate and 40% molar cholesterol) with various basic proteins. (a) With the myelin basic protein, $D = 98$ Å. (b) With cytochrome *c*, $D = 124.9$ Å. (c) With polylysine, $D = 72.5$ Å.

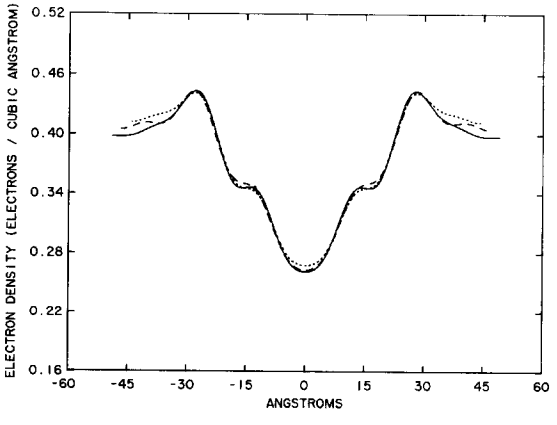

Fig. 10. Electron density profiles for membranes containing the myelin basic protein at different relative humidities. The lipid bilayer is composed of cerebroside sulphate and 40% (molar) cholesterol. The relative humidities are 100% (solid line), 97% (dashed line) and 88% (dotted line).

The structure of bilayers of cerebroside sulphate, cholesterol and myelin basic protein

A typical diffraction pattern recorded from multilayers containing the myelin basic protein is shown in Fig. 9a. It shows eight sharp lamellar reflections with a repeat period of 98 Å. This corresponds to a resolution of about 12 Å, which was typical for the patterns we recorded. We made specimens containing normal and brominated cholesterol and recorded data at 100%, 97% and 88% relative humidities. (As with the lipids alone, the structure became disordered below 88% relative humidity and the lamellar reflections broadened markedly.) Electron density profiles from a swelling series for membranes containing normal cholesterol are shown in Fig. 10. It can be seen that the overall bilayer structure remains constant over this range of water contents although significant changes do occur in the regions between neighbouring bilayers. The distance across the bilayer between the electron-dense headgroups is almost exactly the same as it is in the bilayer of the lipids alone (see Figs. 6 and 7). A comparison between membranes with normal and brominated cholesterol is shown in Fig. 11.

When the profile for the membrane containing the myelin basic protein is compared to that for

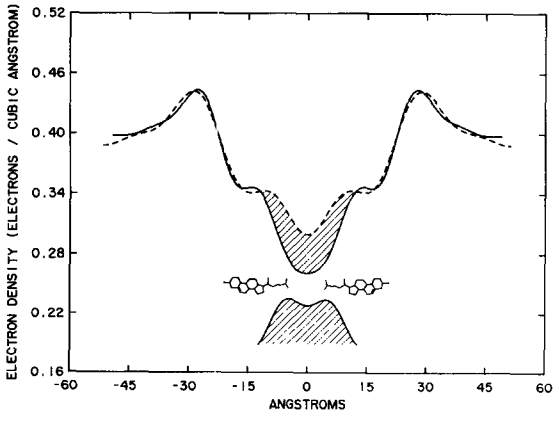

Fig. 11. Electron density profiles for membranes containing the myelin basic protein. The lipid bilayer is composed of cerebroside sulphate and 40% (molar) normal cholesterol (solid line) or brominated cholesterol (dashed line). The relative humidity is 100%. The difference electron density profile shown below represents the distribution of the bromine atoms and thus the positions of the terminal methyl groups of the cholesterol molecules.

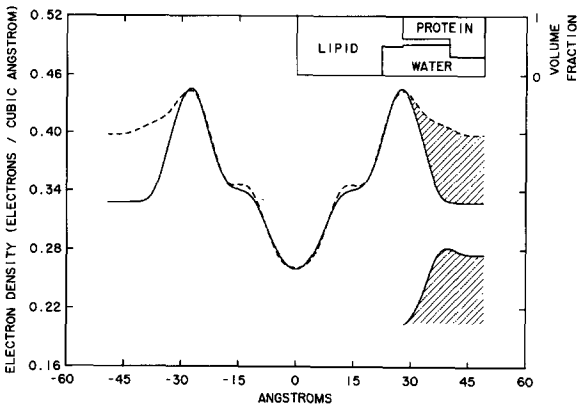

Fig. 12. A comparison between a membrane containing the myelin basic protein (dashed line) and the lipid bilayer alone (solid line). The difference electron density profile shown below represents the distribution of the protein. A representation of the protein-containing membrane in terms of a volume fraction distribution of the various components is shown at the top of the figure. (See text for details.)

the lipids alone (see Fig. 12), the obvious and most striking difference is the much higher level of electron density on the external surfaces of the lipid bilayer. The difference between the two profiles gives the distribution of protein, and this is shown in the lower right-hand-side of the figure; it is not significantly different from zero over the region across the bilayer between the lipid headgroup peaks. The area under the difference profile represents the number of electrons in the protein which are in excess of those in an equivalent volume of water. Thus, knowing the mass [34] and electron [35] densities for the protein (1.387 g · cm^{-3} and 0.441 electrons per Å^3, respectively) and taking the mass density and electron density of water to be unity and 0.334 electrons per Å^3, respectively, we calculate the protein content to be 34% of the total dry weight. This compares well with our directly measured protein content of 30.1 ± 1.1% (mean ± standard error; $n = 8$). It is clear from the profiles in Fig. 12 that there is no significant penetration of protein beyond the position of the sulphate group of the lipid molecule so that within the region between the main peaks, only the lipid and water contribute to the electron density. Similarly, in the lipid profile, a point is reached (at about 40 Å) beyond which the electron density remains close to that of water and

where the lipid does not significantly contribute to the electron density. This can be taken to define a region in the profile for the protein-containing membrane where only water and protein are present. If we assume that water does not penetrate beyond a point defined by the hydrocarbon partial thickness [23] (see Fig. 7) then this defines a region in the profiles (between ± 22.2 Å) where only the lipid contributes. These considerations define four regions in the bilayer between which the various components, protein, lipid and water can be distributed according to the volumes they occupy. (In these calculations we have assumed that, in the region occupied by only lipid and water, these components are distributed in the same proportion as they are in an equivalent region in the bilayer of total myelin lipids [27].) These volumes can be calculated knowing the mass densities and the observed ratio of the masses of the components in the entire structure. This volume fraction representation is shown at the top of Fig. 12.

The structure of bilayers of cerebroside sulphate, cholesterol and cytochrome c

We recorded diffraction patterns from multilayer samples containing cytochrome *c* with both normal and brominated cholesterol, over a range of relative humidities. The patterns were qualitatively the same as those we had recorded from the multilayers containing the myelin basic protein, although the lamellar repeat period was considerably larger (typically 125 Å) and the reflections extended to slightly higher resolution (about 11 Å). Our best diffraction pattern is shown in Fig. 9b. A comparison between electron density profiles for membranes with normal and brominated cholesterol is shown in Fig. 13. The difference profile reflecting the distribution of the bromine atoms is shown in the lower part of the figure. The addition of cytochrome *c* to a bilayer of the lipids greatly changed the observed electron density profile. Profiles for bilayers with and without cytochrome *c* are shown in Fig. 14. As with the myelin basic protein, large changes are evident at the bilayer surfaces although, once again, there is no significant penetration of protein beyond the sulphate group of the lipid molecule. A volume fraction representation of the distribution of protein, lipid and water was derived, based on the same considerations discussed in the previous section, and is shown at the top of Fig. 14. Clearly the distributions of cytochrome *c* and the myelin basic protein are very different (see Figs. 12 and 14); a detailed comparison between the two proteins and their interactions with lipids will be made in Discussion. The area under the difference profile in Fig. 14 corresponds to a protein content of about 40% of the total dry weight (taking the mass [36] and electron [19]) densities for cytochrome *c* to be 1.374 $g \cdot cm^{-3}$ and 0.438 electrons per $Å^3$, respectively) which compares reasonably well with our directly measured protein content of $44 \pm 1.4\%$ (mean $\pm$ standard error; $n = 4$). Some multilayer specimens were prepared on glass microscope slides so that absorption spectra could be recorded. We found that the absorption spectrum (not shown) of a multilayer specimen containing cytochrome *c* was essentially identical to that of cytochrome *c* in aqueous solution.

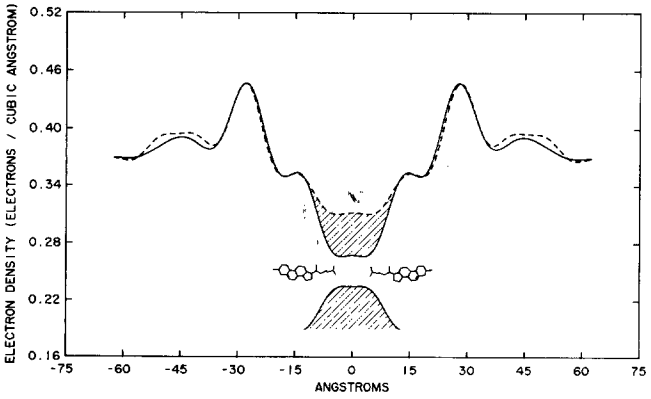

Fig. 13. Electron density profiles for membranes containing cytochrome *c*. The lipid bilayer is composed of cerebroside sulphate and 40% (molar) normal cholesterol (solid line) or brominated cholesterol (dashed line). The relative humidity is 100%. The difference electron density profile shown below represents the distribution of the bromine atoms and thus the positions of the terminal methyl groups of the cholesterol molecules.

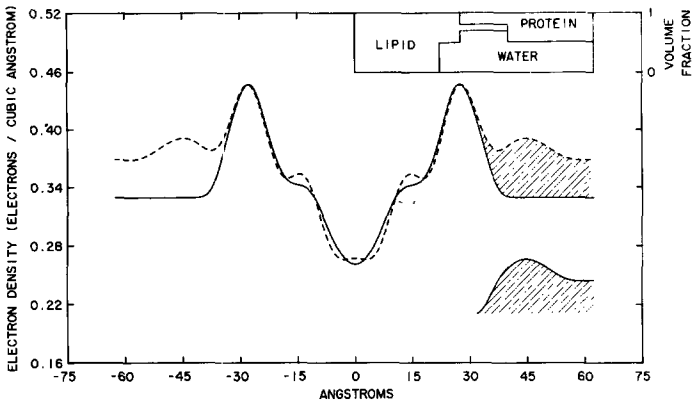

Fig. 14. A comparison between a membrane containing cytochrome *c* (dashed line) and the lipid bilayer alone (solid line). The difference electron density profile shown below represents the distribution of the protein. A representation of the protein-containing membrane in terms of a volume fraction distribution of the various components is shown at the top of the figure. (See text for details.)

The structure of bilayers of cerebroside sulphate, cholesterol and polylysine

We made multilayer specimens containing both polylysine and polyarginine and recorded diffraction patterns from bilayers containing both normal and brominated cholesterol at a variety of relative humidities. The repeat periods were very similar to the repeat periods of the lipids alone, as was the resolution of the diffraction patterns (typically about 6 Å). The specimens with polyarginine (mean molecular weight 44000 $g \cdot mol^{-1}$) all showed more than one series of lamellar reflections indicating phase separation. With polylysine (mean molecular weight 17000 $g \cdot mol^{-1}$), however, only a single lamellar phase was observed (see Fig. 9c). The derived electron density profile, together with that for the lipids alone, is shown in Fig. 15. The chemical analysis of these multilayer specimens showed that they contained a small but significant amount of polypeptide (a few percent of the total dry weight) and this is reflected in the electron density profiles by an increase in density on the external surfaces of the lipid bilayer. Other differences are apparent over the cholesterol nucleus region. Although small, these could be significant, but they are difficult to interpret. The presence of the polypeptide, however, has no significant effect on the distance across the bilayer between the sulphate groups of the cerebroside sulphate molecule.

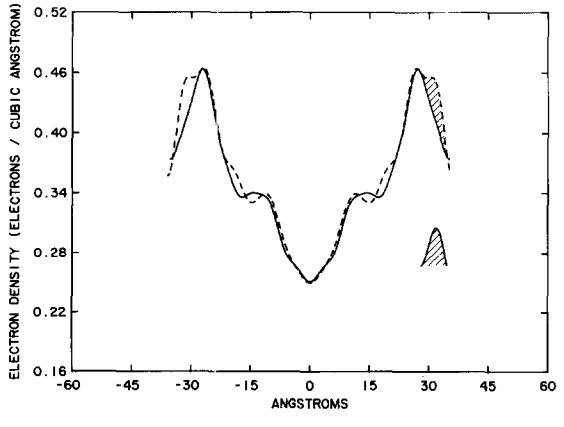

Fig. 15. A comparison between high resolution electron density profiles for a lipid bilayer in the presence (dashed line) and absence (solid line) of polylysine. The lipid bilayer was composed of cerebroside sulphate and 40% (molar) cholesterol and the shaded region is interpreted as showing the distribution of the polypeptide.

Discussion

The structure of a cerebroside sulphate / cholesterol bilayer

The fact that a mixture of cerebroside sulphate and 40% molar cholesterol forms a well-ordered multilayered stack which takes up only a limited amount of water at 100% relative humidity is, in itself, an interesting observation. This behaviour has been reported previously for other charged lipids which swell indefinitely in excess water yet form ordered multibilayer stacks in equilibrium with water vapour (see Ref. 27). Whatever the reason for this behaviour it allows relatively high resolution X-ray diffraction patterns to be recorded which can provide a detailed description of the bilayer structure. The bilayer thickness, as measured by the distance between the

electron-dense peaks (see Fig. 7), is considerably larger than that for total myelin lipids (55.5 Å compared with 46 Å [27]) although the average hydrocarbon chain length differs only slightly (18.1 for total myelin lipids and 18.8 for cerebroside sulphate [26]). The major differences evidently lie in the extended cerebroside sulphate headgroup and the more saturated hydrocarbon chains.

The cholesterol molecule is positioned in the bilayer such that its hydroxyl group is close to the hydrocarbon/water interface. With the hydroxyl group effectively anchored at the interface, the terminal methyl groups of the cholesterol molecules are about 9 Å apart. This is reflected in the difference profile of Fig. 7 which shows a relatively broad distribution of the bromine atoms. This is in contrast to dimyristoylphosphatidylcholine (14 carbons per chain) and cholesterol bilayers [15] in which the terminal groups of the cholesterol molecules are much closer together and give a much sharper bromine difference profile. In the cerebroside sulphate/cholesterol bilayer, the region at the centre of the bilayer between the cholesterol molecules must be filled by hydrocarbon chains from adjacent cerebroside sulphate molecules or those from molecules in the opposing monolayer. Presumably the variable, and on average much longer, hydrocarbon chain of the cerebroside sulphate molecule either folds around to pack beneath the cholesterol molecule or extends and interdigitates across the bilayer centre. The relatively high level of electron density at the bilayer centre of 0.252 electrons per $Å^3$ (see also Ref. 27) is consistent with considerable interdigitation (the electron density of a methylene group is 0.296 electrons per $Å^3$ while that of a methyl group is 0.167 electrons per $Å^3$ [31]). This is again in sharp contrast with dimyristoylphosphatidylcholine and cholesterol where the electron density at the bilayer centre is close to that of a methyl group and where, presumably, little interdigitation is occurring.

The interactions between basic proteins and lipids

Proteins and polypeptides which are rich in basic residues, such as the myelin basic protein, cytochrome *c* and polylysine, are known to interact strongly with acidic lipids but only weakly with uncharged lipids. In a selection of acidic lipids, however, the one which appears to interact most strongly depends upon both the parameter being measured and the technique being used (see. e.g. Refs. 17, 37 and 38). Consistent with this strong interaction, we found that the myelin basic protein, cytochrome *c* and polylysine all bound tightly to a cerebroside sulphate and cholesterol monolayer and produced excellent Langmuir-Blodgett specimens.

Although the strength of the interaction is not in dispute, the effects that basic proteins and peptides have on bilayer structure, and their detailed location in the lipid bilayer, are contentious issues. Many workers have studied the interactions between the myelin basic protein and lipid vesicles, and there is little doubt that the predominant forces acting are electrostatic in nature. However, results on the effects of the myelin basic protein on vesicle permeability to glucose [7] and sodium ions [8], phase transition temperatures [8,9] and hydrocarbon chain motion [9] have been interpreted as reflecting a hydrophobic interaction between the lipid hydrocarbon chains and certain segments of the myelin basic protein which have been postulated to penetrate into the hydrocarbon core of the bilayer (for reviews, see Refs. 5 and 6). This interpretation has been supported by work on lipid monolayers which has shown that, when the myelin basic protein is injected beneath a monolayer of acidic lipids, the monolayer pressure increases [10]; this increase in pressure was interpreted as a partial penetration of the myelin basic protein into the lipid monolayer. Moreover, certain regions of the protein appear to be protected from proteolytic cleavage when bound to a monolayer [16] or lipid vesicles [39] and this has also been taken as support for the notion that the myelin basic protein partially penetrates the bilayer. While the individual lines of evidence are not wholely convincing, and a priori reasoning argues against small loops of polypeptides being sufficiently apolar to penetrate the lipid hydrocarbon region, nonetheless, taken together, the weight of evidence appears to favour some sort of hydrophobic interaction between the myelin basic protein and lipids. The evidence has, however, been indirect in nature and there have been very few attempts to study the interactions between the myelin basic protein and lipids with direct struct-

ural techniques. In an early investigation, Mateu et al. [11] studied the structure of lamellar phases of a mixture of acidic lipids and the myelin basic protein and localized the protein on the membrane surface. They suggested that the protein was associating preferentially with the sulphatides. More recently, Sedzik et al. [12] derived possible electron density distributions for bilayers of myelin lipids containing the myelin basic protein. They were not able, however, to deduce a unique scale factor between the profile for the lipids alone and that for the bilayer containing the protein. While concluding that the protein was located predominantly between bilayers, the degree of penetration into the headgroup region was uncertain, but as much as one third of the total protein may have been inserted between the lipid headgroups. They did conclude that the lipid bilayer thickness was little affected by the presence of the myelin basic protein. A brief report by Murthy et al. [13] compared the interactions of the protein and polylysine with bilayers of phosphatidyl glycerol. Their diffraction data, however, were of even lower resolution than that of Sedzik et al., and they were also unable to rigorously determine scale factors when comparing their electron density profiles. Despite these problems, they nonetheless uneqivocally concluded that the myelin basic protein did penetrate the lipid bilayer.

Although the determination of an accurate scale factor between an electron density profile for lipids alone and that for a protein-containing membrane is essential for any diffraction analysis of this sort, this is not the only problem which can hinder the interpretation of an electron density profile. Equally important is an accurate determination of the ratio of protein to lipid in the specimen together with a knowledge that the diffraction specimen is homogeneous in terms of the distribution of protein and lipid. For example, the method of specimen preparation employed by Sedzik et al. (myelin basic protein was added to sonicated lipid vesicles) may result in vesicles with differing amounts of protein bridging the spaces between adjacent bilayers. For example, with cytochrome *c* and phospholipids, multilamellar phases have been described which have either one or two protein molecules incorporated between the lipid bilayers [40–42]. The methods of specimen preparation and chemical analysis employed here, together with our determination of an absolute electron density scale, largely circumvents these problems. The difference electron density profile for the myelin basic protein (Fig. 12) shows clearly that there is no measurable penetration of the protein into the hydrocarbon region or indeed, beyond the position of the sulphate group of the lipid molecule. Consistent with previous work [12] we find that the protein has little or no effect on the lipid bilayer structure itself, although a considerable fraction of the protein may penetrate into the headgroup region (see also Ref. 43), perhaps, as suggested by Sedzik et al. [12], into the spaces above the cholesterol molecules on the surface of the bilayer.

Although the results found here are for one bilayer system only (cerebroside sulphate and cholesterol) and the myelin basic protein quite clearly interacts differently with different lipid species (for reviews, see Refs. 5, 6), it should be emphasised that some of the most persuasive evidence in favour of a hydrophobic interaction has been obtained using cerebroside sulphate [10,16]. Indeed, the expansion of monolayers by the myelin basic protein was particularly large with cerebroside sulphate, and this effect has been observed in the present work. However, when a bilayer is formed, we find no evidence that the protein penetrates the hydrocarbon core. The danger of inferring details about bilayer structure from the behaviour of monolayers is clear, particularly when the monolayer pressure is low and the lipids are packed very differently from those in bilayers. It is worth noting that the apparent penetration into the monolayer invariably decreases as the monolayer surface pressure increases [10] and as the lipids approach a state more akin to that found in bilayers.

Interestingly, while cytochrome *c* and the myelin basic protein both expand monolayers, polylysine causes a reduction in monolayer pressure. In all three cases, however, we find there is no penetration of the protein or polypeptide into the lipid bilayer (see Figs. 12, 14 and 15) beyond the position of the sulphate group. The contrasting effects of proteins and polypeptides on monolayer surface pressure could well be due to their different charge densities, with the high density of basic residues on the polypeptides tending to 'condense' the lipid

headgroups whereas the increased separation of the basic groups on the proteins tends to increase the distance between the lipid molecules. In view of the results we have obtained, it seems likely that the previously observed effects of basic proteins on monolayers and bilayers containing acidic lipids would be more plausibly interpreted in terms of pure electrostatic interactions with the lipid headgroups which then result in secondary effects on the hydrocarbon chains.

The structure of the myelin basic protein

In solution, the myelin basic protein appears to have a fair degree of secondary structure although no specific regions of alpha helix or beta sheet. Most work (see Refs. 5,6, for review) is consistent with a structure having an unusually large axial ratio (approximately 10 : 1) with dimensions around 150 Å by 15 Å [44]. There is, however, some evidence that the protein changes its structure when interacting with lipids or detergents and, in particular, forms definite regions of alpha helix and beta sheet [45,46]. Nonetheless, a previous X-ray study concluded that the myelin basic protein has similar dimensions both in solution and when associated with lipids [47].

The X-ray diffraction results presented here show clearly that the protein has one relatively narrow dimension of about 15 Å perpendicular to the membrane surface (see Fig. 12). This dimension is much larger than the 5 Å found for polylysine (see Fig. 15), which must be completely extended on the bilayer surface, and is consistent with the myelin basic protein having a considerable degree of secondary structure. From our observed protein content and the known unit cell area we can calculate that in the membrane plane the protein extends to cover an area of about 2500 $Å^2$ which is entirely consistent with a structure whose in-plane dimensions are 15×150 $Å^2$, although other structures in the membrane plane are, of course, possible. The protein covers an area occupied by about 38 cerebroside sulphate molecules; this can be compared to the number of lipid molecules specifically interacting with each protein molecule, which appears to be about 26 (taking an average of several estimates [48–51]). The folding of the protein might be such that most of the 31 positively charged amino acid residues interact with the negatively charged headgroups of the lipid molecules. The very close association of the protein with the lipid headgroup region certainly supports this picture. Indeed, over 40% of the protein is in a region also occupied by the lipid headgroups.

This is in marked contrast to the distribution of the cytochrome *c* molecule in the cerebroside sulphate/cholesterol bilayer. Although obviously interacting electrostatically with the lipid headgroups, only 18% of the protein lies within the headgroup region. The overall dimension of the difference electron density profile of about 30 Å, and the projected area in the membrane plane of about 900 $Å^2$ (calculated from the observed protein/lipid ratio and known unit cell area) is consistent with an intact folded protein molecule (whose dimensions are known [52] to be that of a prolate spheroid $30 \times 34 \times 34$ $Å^3$) lying on the bilayer surface with its short dimension perpendicular to the bilayer plane (see also Ref. 53). Our finding that the absorption spectrum in the multilayers is essentially identical to that found in solution is consistent with the protein maintaining its native structure. Our observation that cytochrome *c* can bind on the surface of bilayers which contain acidic lipids and yet have little or no effect on membrane structure, is one that has been made previously by several workers [12,40–42], although Sedzik et al. [12] were the first to contrast the differences between cytochrome *c* and the myelin basic protein.

A particularly interesting feature of the difference electron density profile for the myelin basic protein (Fig. 12) is the relatively high level of electron density at the interface between neighbouring membranes. Such a feature suggests some degree of specific interaction between the protein molecules in adjacent membranes. The possibility that the myelin basic protein molecules form stable dimers has long been considered, and support for this idea has come from observations of dimerization is solution and in the intact myelin sheath [54–56]. More recently, it has been demonstrated that the protein can form multibilayer vesicles by protein-protein dimerization across the space between neighbouring membrane surfaces [50] and that the proteins interact in the presence of lipid with a dissociation constant of about 1

mM [57]. Using this dissociation constant, we can calculate that, under our conditions, a negligible proportion of dimers would exist before the monolayers were transferred to the solid support. Once the bilayers had been formed, however, essentially all the protein molecules would be in the form of dimers. The relatively high level of electron density we see at the membrane-membrane interface could reflect this formation of protein-protein dimers.

In the intact myelin sheath, the basic protein is probably located in the cytoplasmic space between membrane pairs [4,5,43] but whether or not protein dimers form across the boundary is equivocal. Indeed, although the distance between neighbouring bilayers across the cytoplasmic boundary in myelin is rather variable between various species (23–30 Å; [32]), the equivalent distance across the aqueous region in our membrane stacks (42 Å; see Fig. 12) is substantially larger. It is also worth noting that despite the very strong interactions that are observed between cerebroside sulphate and the myelin basic protein in vitro, there is no evidence that such interactions occur in vivo. Moreover, the protein/cerebroside sulphate ratio in our membranes is considerably larger than that found in myelin so we would be cautious before interpreting our results directly in terms of the structure of the intact myelin membrane. In the context of the vast literature on the interactions between the myelin basic protein and lipids, however, our principal conclusions are unambiguous. The myelin basic protein interacts strongly with a bilayer of cerebroside sulphate and cholesterol in the form of an extended molecule covering an area of 2500 $Å^2$ in the plane of the membrane and having a dimension perpendicular to the membrane plane of about 15 Å. The protein interacts intimately with the headgroups of the lipid molecules but does not penetrate into the hydrocarbon region of the lipid bilayer.

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