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A HYDROCARBON-SOLUBLE PROTEIN–LIPID COMPLEX: CHARACTERIZATION BY CHEMICAL ANALYSIS, ULTRACENTIRFUGATION AND ELECTRON MICROSCOPY*

W. LESSLAUER**, F. C. WISSLER AND D. F. PARSONS

Biophysics Department, Roswell Park Memorial Institute, Buffalo, N.Y. 14203 (U.S.A.)

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SUMMARY

A protein extracted from myelin is shown to form a complex with phospholipids which is soluble in hydrocarbons. Complex formation is suggested by the solubility characteristics in hydrocarbon solvents of the protein with and without phospholipids. These complexes appear as defined entities on the basis of chemical analysis and the behaviour in the analytical ultracentrifuge. Electron microscope studies yield some insight as to how protein and phospholipid molecules may be assembled in the complex. The complex is treated and discussed strictly as a model system.

INTRODUCTION

The chemical composition of some biological membranes is known with accuracy. The mutual geometric arrangement of the different components, however, is open to controversy. Yet, there is every reason to believe that the proper functioning of membranes and membrane-linked enzyme systems is closely related to the assemblage of a small number of molecules into a specific microenvironment.

Proteins and lipids are main constituents of membranes and their interaction may be important in consolidating their structure. Protein–lipid interaction has been studied in model systems such as monomolecular lipid films (*e.g.* refs. 13 and 16), black lipid membranes (*e.g.* refs. 9, 14 and 17) and phospholipid vesicles (*e.g.* refs.11, 12 and 15). A hydrocarbon-soluble complex of cytochrome *c* and various phospholipids was described by DAS, HAAK AND CRANE[8]. These model systems depend upon the tendency of amphipathic molecules such as phospholipids to assemble in an ordered manner and to create interfaces between phases of different polarizability. Such interfaces provide insight into how ordered structures can be organized at the molecular level.

As will be reported below, a membrane protein extracted from myelin can be shown to form a complex with phospholipids which is soluble in hydrocarbons[18].

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** Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

This complex is a defined entity and can be characterized by different methods, such as chemical analysis, ultracentrifugation and electron microscopy. The appearance in the electron microscope is compared to that of the analogous cytochrome *c*–phospholipid complex.

MATERIALS AND METHODS

The protein, which will be called 'myelin protein', was isolated from beef brain white matter after the method of LOWDEN, MOSCARELLO AND MORECKI[4]. The purity of the preparation was checked by electrophoresis on urea–starch gel and by ultracentrifugation[4].

Phospholipids were isolated from natural sources and not used unless shown to be homogeneous by thin-layer chromatography.

All organic solvents were redistilled before use.

Complexes of the myelin protein and phospholipids were prepared in a standardized procedure. 15–20 mg phospholipid were dispersed and sonicated in 10 ml distilled water, 4.0 ml ethanol, 5–10 mg myelin protein and 14 ml hydrocarbon (hexane, isooctane or decane) were subsequently added. The preparation was shaken vigorously for 1 h at room temperature, and the aqueous and hydrocarbon phases were separated.

The protein concentration was determined after the method of LOWRY *et al.*[3]. Phospholipids were determined as phosphorus after HANES AND ISHERWOOD[1] and CHEN *et al.*[2].

Ultracentrifugation experiments were carried out in a Beckman type E analytical ultracentrifuge with a titanium rotor. Regular, synthetic boundary and partition cells were employed. Sedimentation coefficients in aqueous or in hydrocarbon phase were obtained from the Schlieren patterns. Diffusion coefficients were calculated according to the area–height method.

Optical rotatory dispersion curves of the myelin protein in 0.1 M NH_4Cl and in 8 M urea were obtained on a Japan Spectroscopic Co. instrument.

The complex of the myelin protein and phospholipids precipitated out of the hydrocarbon phase when OsO_4 (0.5–1 %), dissolved in hexane, was added. The precipitate was collected and directly embedded in Epon 812. Complexes in water–hydrocarbon systems were fixed with OsO_4 (Palade's buffered OsO_4 fixative) or with $KMnO_4$ (Luft's buffered permanganate fixative), dehydrated with acetone and embedded in Epon 812. Sections were double stained with uranyl acetate and lead citrate. The specimens were sectioned on a LKB ultramicrotome. Electron micrographs were taken on a Philips EM 300 electron microscope.

RESULTS

The myelin protein preparation in 0.1 M NH_4Cl (pH 2.3) exhibited a single peak in sedimentation velocity experiments in the analytical ultracentrifuge, when viewed with the Schlieren optical system[4]. The sedimentation coefficient was $s_{20,w} = 1.1$ S. The Schlieren pattern was used to calculate a diffusion coefficient of the myelin protein after the area–height method ($D = 4.2 \cdot 10^{-7}$ cm^2/sec). The molecular weight determined from $s_{20,w}$ and D was 24 600, if the partial specific volume was put at

$\bar{\nu} = 0.74$. This agrees fairly well with the value of 22 800 obtained from sedimentation equilibrium[5]. The ratio of the observed frictional coefficient in the ultracentrifuge experiments and the frictional coefficient of an ideally spherical molecule was calculated and found to be $f/f_0 = 2.7$.

The optical rotatory dispersion curve of the protein was identical in 0.1 M NH_4Cl and in 8 M urea. Optical rotatory dispersion curves were evaluated after MOFFITT's theory[19]. The parameter thought to be proportional to the content in α-helix of a protein molecule was found to be $b_0 = 0$.

When an aqueous phase containing myelin protein and phospholipids was shaken with a hydrocarbon phase and the two phases then were separated and analyzed, it was found that both protein and phospholipid were present in the aqueous and hydrocarbon phases (Table I). The hydrocarbon solubility was attributed to a complex formation with phospholipids. Such complexes were formed of myelin protein

TABLE I

SEDIMENTATION COEFFICIENTS, DIFFUSION COEFFICIENTS AND RELATIVE AMOUNTS OF PROTEIN AND PHOSPHOLIPID IN THE HYDROCARBON PHASE FOR THE COMPLEX PREPARATIONS

Average values are given; see text for number of experiments and range of variation.

	$s_{20,\,isooctane}$ (S)	D (cm^2/sec)	*Relative amounts in hydrocarbon phase*	
			Protein	*Phospholipid*
Phosphatidylethanolamine complex	6.3/15.4	$2.05 \cdot 10^{-6}/1.25 \cdot 10^{-6}$	0.27	0.76
Lecithin–cardiolipin complex	14.7/(19.2)	$0.95 \cdot 10^{-6}$	0.18	0.20

and either phosphatidylethanolamine or mixed lecithin–cardiolipin. The protein alone, under the same experimental conditions, was not soluble in hydrocarbons. Phosphatidylethanolamine in the absence of protein distributed between the two phases (relative concentration of phosphatidylethanolamine in the hydrocarbon phase approx. 0.3); whereas mixed lecithin–cardiolipin without protein was, under the experimental conditions, nearly insoluble in the hydrocarbon phase (relative concentration of lecithin–cardiolipin in the hydrocarbon phase less than 0.01)[8]. The lecithin to cardiolipin ratio was 1:1.5 at the beginning of the preparation. The lecithin–cardiolipin complex in isooctane dissociated when applied to a thin-layer plate (Silica G). The plate was developed in the usual way, and the relative amounts of lecithin and cardiolipin were estimated. No preferential incorporation of either lecithin or cardiolipin into the complex was detected within the limits of this method. The molar ratio of protein to phospholipid in the hydrocarbon phase, based on protein and phosphorus determinations, was 1:75 (± 25) for the lecithin–cardiolipin complex and 1:160 (± 50) for the phosphatidylethanolamine complex (arithmetic average of 4 and 5 independent preparations of the lecithin–cardiolipin and phosphatidylethanolamine complex, respectively). The yield of the complex preparations, as determined by the relative amount of protein to be found in the hydrocarbon

phase, varied considerably from experiment to experiment (Table I). Deviations of up to ± 40 % of the average values in Table I were observed.

The lecithin–cardiolipin and phosphatidylethanolamine complexes in isooctane were studied in the analytical ultracentrifuge at or near 20°. With the phosphatidylethanolamine complex preparations, two peaks were observed in the Schlieren pattern (Fig. 1). The sedimentation coefficients in isooctane, corrected for 20° were $s_{20,isooctane}$ = 6.3 (± 0.3) S and 15.4 (± 1.0) S (average of 5 experiments) in the range of concentrations of 0.4 to 1.7 mg protein/ml isooctane. Diffusion coefficients were calculated from the Schlieren pattern following the area–height method .This method yielded values of $2.05 \cdot 10^{-6}$ cm²/sec for the 6.3-S peak and $1.25 \cdot 10^{-6}$ cm²/sec for the 15.4-S peak. Over the observed range, the diffusion coefficients were practically independent of the concentration and reproducible within ± 5 %. The 6.3-S peak was separated in a partition cell. Chemical analysis showed that it contained phosphatidylethanolamine alone and no protein. With the lecithin–cardiolipin complex (Fig. 2), one peak with $s_{20,isooctane}$ = 14.7 (± 0.6) S (average of 3 experiments) was always observed at concentrations of 0.5–1.2 mg protein/ml isooctane. In one experiment a second peak with $s_{20,isooctane}$ = 19.2 S (concentration 1.05 mg protein/ml isooctane) appeared. The diffusion coefficient of the 14.7-S peak obtained from the area–height method was $0.95 \cdot 10^{-6}$ cm²/sec. The partial specific volume of the complex in isooctane was determined as 0.70.

In the electron microscope the morphological appearance of the phosphatidylethanolamine and lecithin–cardiolipin complexes was basically the same. When fixed with OsO_4 in the hydrocarbon phase the complexes appeared aggregated in irregular clusters (Fig. 3). The complex preparation in Fig. 3 was fixed for 30 min in an OsO_4 solution of 0.5 % final concentration. Electron micrographs of preparations with longer fixation times of 12 h or more showed electron dense, nearly spherical

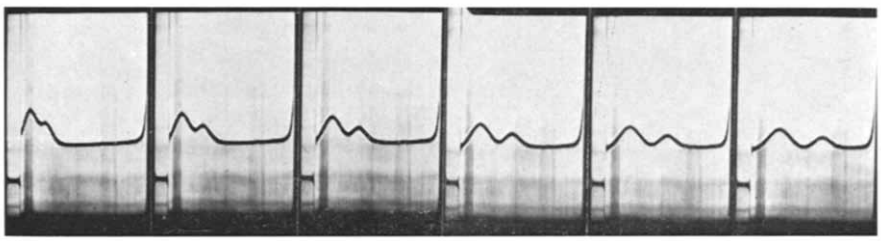

Fig. 1. Schlieren pattern of phosphatidylethanolamine complex preparation in isooctane. 42040 rev./min, 19.3°, 8-min intervals between photographs.

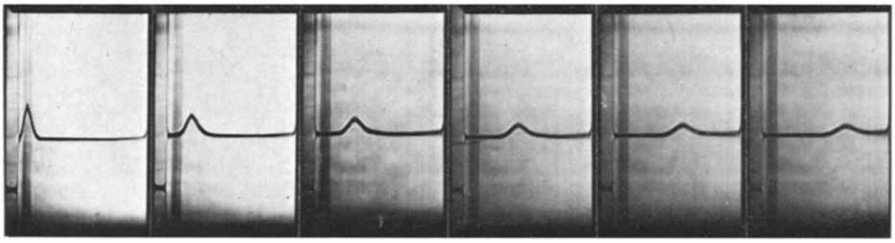

Fig. 2. Schlieren pattern of lecithin–cardiolipin complex preparation in isooctane. 52640 rev./min, 19.0°, 8-min intervals between photographs.

particles in these clusters (see insert in Fig. 3). The diameter of these particles was 35–40 Å, and their center to center distance was not less than 60 Å.

Rather stable emulsions were formed upon shaking the hydrocarbon phase with water or an aqueous solution, which could be separated again only by centrifugation at high speeds (30000 × *g*). The complex lined up at the interface and stabilized the emulsion. The material at the interface could be fixed with OsO_4 or $KMnO_4$. It was further prepared for electron microscopy as if it were a tissue (*i.e.*, dehydrated with acetone and embedded in Epon 812). Electron micrographs of

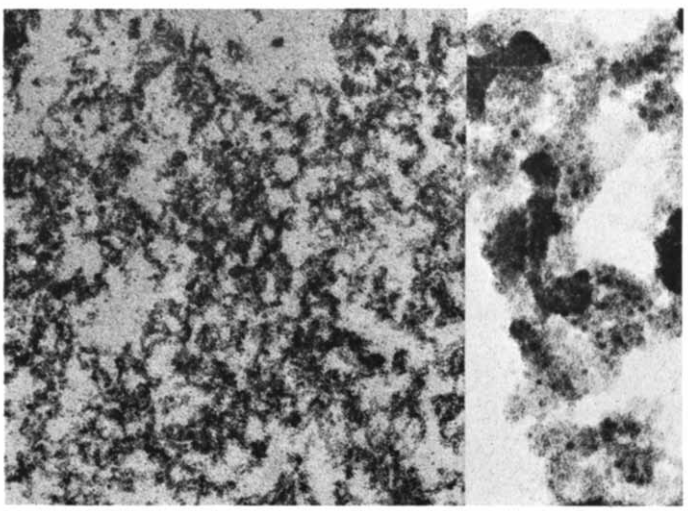

Fig. 3. Electron micrograph of lecithin cardiolipin complex and phosphatidylethanolamine complex (smaller insert) in isooctane, 30 min and 12 h in 0.5% OsO_4, respectively. Sections were stained with uranyl acetate and lead citrate. Magnification 68000 × and 238000 ×.

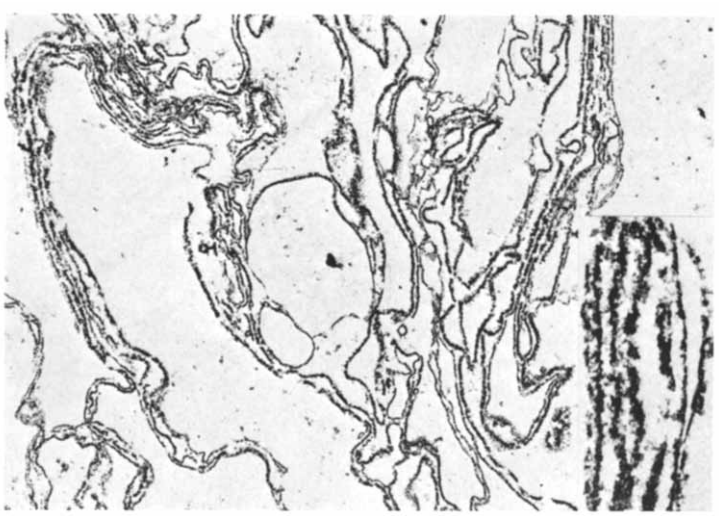

Fig. 4. Electron micrograph of lecithin–cardiolipin complex at water–hydrocarbon interface, 60 min $KMnO_4$. Sections stained with uranyl acetate and lead citrate. Magnification 11900 × and 38400 × (insert).

this material showed extended, sheet-like arrays of the lined-up complex. The thickness of this interfacial membrane was 80–120 Å (Figs. 4 and 5). Its structure was not symmetrical and two sides could be distinguished. One side was a sharply defined and smooth line; the other side had a more irregular structure (Fig. 5). Isolated clusters comparable to those of Fig. 3 were adhering to the irregular side.

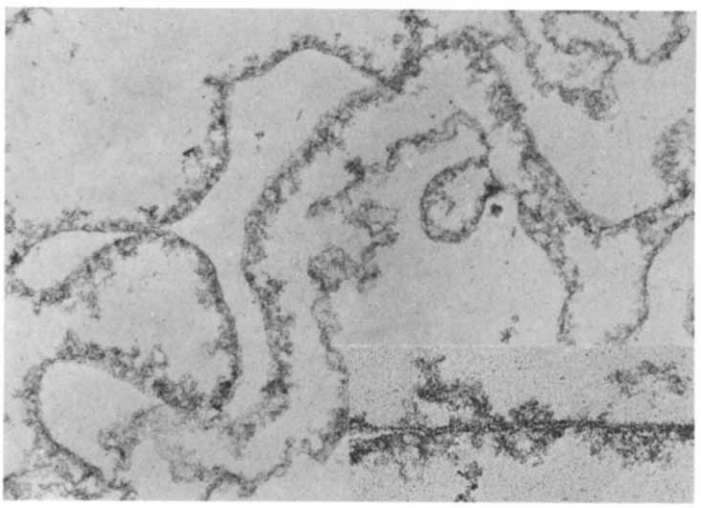

Fig. 5. Electron micrograph of phosphatidylethanolamine complex at water–hydrocarbon interface, 60 min OsO_4. Sections stained with uranyl acetate and lead citrate. Magnification 40900 × and 74000 × (insert).

DISCUSSION

Model systems can be discussed in a more precise way if its components are homogeneous materials. The method of LOWDEN *et al.*[4] enables a pure protein to be obtained from myelin membranes. The preparation is homogeneous by electrophoresis on urea–starch gel and by ultracentrifugation[4]. This protein makes up approx. 10% of the total membrane proteins[4]. No enzymatic activity has been reported. It is a basic protein as shown by the electrophoretic behaviour and the amino acid analysis[4].

The preparation procedure involves treatment with 0.2 M H_2SO_4 and a certain degree of denaturation must be suspected. The optical rotatory dispersion of the protein and the evaluation of the MOFFITT equation suggests zero or a very low content of α-helix. This, as well as the identical optical rotatory dispersion curves in 0.1 M NH_4Cl and in 8 M urea solutions, may indicate that the protein is denatured, if it is assumed that it contains some α-helix in the native state. The calculated ratio of frictional coefficients excludes a globular structure of the protein molecule.

The observation that the myelin protein in the presence of phospholipids is soluble in a hydrocarbon phase can be explained by the assumption that a complex of protein and phospholipid is formed. Such a complex would be made up of a protein molecule surrounded by a layer of phospholipid molecules with their polar groups pointing inside towards the protein and their fatty acid chains oriented towards the hydrocarbon phase. Although no stoichiometric relation, in the strict sense,

is found between protein and phospholipid, their molar ratio is rather constant and small enough to exclude that the protein is merely dragged along into the hydrocarbon phase by huge inverted lipid micelles of undefined structure. A molar ratio of 1:75 as found for the lecithin–cardiolipin complex corresponds approximately to the amount of phospholipid required for a monomolecular layer around the protein.

The molar ratios are based on the protein and phosphorus determinations of the hydrocarbon phase. They do not reflect true molar ratios of the complex, if not all phospholipid in the hydrocarbon phase is really bound in the protein–phospholipid complex. Mixed lecithin and cardiolipin remains nearly exclusively in the aqueous phase in blank experiments when no protein is present, but otherwise identical conditions. Although this is probably not the equilibrium distribution, one may conclude that with lecithin and cardiolipin most of the phospholipid in the hydrocarbon phase is bound to protein. In preparations of the phosphatidylethanolamine complex, however, this conclusion is not valid. The hydrocarbon phase is readily accessible to phosphatidylethanolamine in the absence of protein. The high molar ratio of 1:160 does not represent the molecular composition of the phosphatidylethanolamine complex, but is due to the presence of phosphatidylethanolamine in free or micellar form not connected with protein, as is shown also by ultracentrifuge experiments with a partition cell. There is evidence that the true molar ratio for the phosphatidylethanolamine complex is nearly the same as for the lecithin–cardiolipin complex.

The formation of such phospholipid complexes seems not to be a general property of proteins. Attempts to obtain analogous complexes with bovine serum albumin have been unsuccessful. A similar complex formed by cytochrome *c* and phospholipids, however, has been described by DAS *et al.*[8]. The presence of basic groups on the protein together with a net negative charge on the phospholipids may be essential requirements.

The observation of one or two well-defined peaks in the analytical ultracentrifuge indicates that the complexes in the hydrocarbon phase exist in one, or in two distinctly different kinds of defined and nearly uniform molecular aggregates. The variation of the sedimentation coefficients represents probably a true variation in the stoichiometric composition of the complex from one experiment to another, despite the attempt to standardize experimental conditions. The diffusion coefficients determined according to the area–height method can be considered at least as good approximations for the correct values. Under the assumption that the composition of the complex may vary from experiment to experiment to some extent, but is uniform in a given preparation, molecular weights of the complexes can be calculated from the sedimentation and diffusion coefficients. The molecular weights for the species giving rise to the 6.3-S and the 15.4-S peaks of the phosphatidylethanolamine complex are 15000–17000 and 63000–68000, respectively. The 14.7-S peak of the lecithin–cardiolipin complex corresponds to a molecular weight of 70000–80000. The once observed 19.2-S peak in a lecithin–cardiolipin complex preparation can be attributed to a polymeric form. Molecular weights of 70000 are consistent with the proposed model of the complex, namely one protein surrounded by phospholipid molecules. It corresponds to a ratio of one protein to 60–65 phospholipid molecules, if the average molecular weight of phospholipids is taken as 750.

The 6.3-S peak in the phosphatidylethanolamine complex preparations cannot

be due to a protein–lipid complex, since the molecular weight of the protein alone is higher than the observed 15000–17000 of the species of this peak. It is due to free phosphatidylethanolamine not connected with protein. This agrees with the observation that phosphatidylethanolamine does not need protein for distributing through both the aqueous and hydrocarbon phases. This was verified by chemical analysis of the 6.3-S peak when it was separated in a partition cell.

A likely interpretation of electron micrographs of the complex in isooctane is that the structure of the complex is preserved during fixation and that the clustering is caused by cross-linking brought about by chemical reaction of OsO_4 and the fatty acid chains[6,7]. The electron dense regions which appear after long fixation times (Fig. 3) may be attributed tentatively to a heavier incorporation of OsO_4 into the central portion of the complex, which takes place at a slower rate than the reaction with the fatty acid chains. However, a complete molecular rearrangement of the complex due to the fixation cannot be excluded. Electron micrographs of the analogous cytochrome *c*–phospholipid complex (Fig. 6) do not show the same clustering in a random pattern as the myelin protein complex. In the cytochrome *c* complex, extended sheet-like arrays or isolated particles are observed[10,20]. This may indicate that the distribution of phospholipids around cytochrome *c* is much more asymmetric than around the myelin protein molecule. The cytochrome *c*–phospholipid complex has been shown to aggregate in polymeric forms even in the absence of any fixing agent such as OsO_4 (ref. 21). The aggregation of the myelin protein and the cytochrome *c* complex in the hydrocarbon phase observed in the electron microscope needs, therefore, not to be a fixation artifact.

The fact that the myelin protein complex is surface active and lines up at hydrocarbon–water interfaces (Figs. 4 and 5) requires that the phospholipid shell is not completely symmetrical and that regions of high polarity are left on the surface of the complex. It cannot be excluded, however, that a complete rearrangement of the complex occurs at the interface and that the complex itself is not amphipathic.

Fig. 6. Electron micrograph of cytochrome *c* complex with lecithin and cardiolipin, in isooctane fixed with OsO_4. Sections stained with uranyl acetate and lead citrate. Magnification 32800 × and 84000 × (insert).

A schematical representation of these two discussed models is shown in Fig. 7.

It is difficult to decide which side of these membrane-like arrays (Figs. 4 and 5) is oriented against the hydrocarbon and which side against the aqueous phases. The minimum observed distance between the smooth sides for two adjacent membrane-like sheets was approx. 80 Å. This spacing could be due to unstained fatty acid chains. The irregular side would then be directed against the aqueous phase. On the other hand, there is evidence that the preparation is an oil-in-water emulsion.

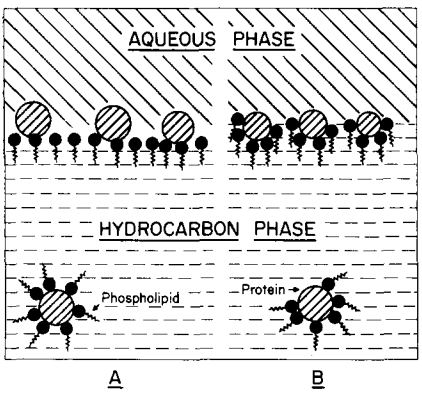

Fig. 7. Complex at the water–hydrocarbon interface (shown schematically). A. Rearrangement of molecular structure at the interface. Monomolecular phospholipid film forms interface, covered by a layer of protein. B. Complex is originally asymmetric and amphiphilic, and lines up at the interface.

The aqueous phase is removed first during the dehydrating procedure. Furthermore, the material loosely adhering to the irregular side is very similar in appearance to electron micrographs of the complex in hydrocarbon phase. A better interpretation may be, therefore, that the smooth side is directed towards the aqueous phase.

While complex formation between protein and phosphilipid in the hydrocarbon phase is obvious, it is very likely that these molecules are not independent from each other in the aqueous phase either, where some form of aggregation between protein and phospholipid micelles is to be expected.

CONCLUSIONS

Current models of the molecular structure of biological membranes imply that regions exist within the membrane which are of hydrocarbon-like nature. These regions are made up of oriented fatty acid chains of phospholipids and of other membrane lipids. Proteins are thought to be bound to these structures by a combination of polar and hydrophobic interactions. They either sit on the membrane surface or are buried in the membrane interior and may form a protein bridge across the membrane.

Biological membranes are without doubt organized in a highly delicate way and the complex of a myelin protein and various phospholipids cannot be regarded as the dominant structural unit of the myelin membrane. Discussed as a model, however, it may show how a protein could be integrated into a biological membrane.

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