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PREPARATION AND PROPERTIES OF VESICLES OF A PURIFIED MYELIN HYDROPHOBIC PROTEIN AND PHOSPHOLIPID A SPIN LABEL STUDY

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SUMMARY

Lipophilin, a hydrophobic protein purified from the proteolipid of normal human brain myelin, was recombined with phosphatidylcholine by solubilization of the lipid and protein in 2-chloro-ethanol followed by dialysis against buffer. This method resulted in homogeneous incorporation of the protein into lipid vesicles as judged by sedimentation on a sucrose gradient and freeze fracture electron microscopy. The lipid-protein vesicles were single layered, 1000–2000 Å in diameter and the freeze fracture faces contained intramembrane particles. The effect of lipophilin on the organization of the lipid was studied by use of spin label probes. Two distinct components were present in the spectrum of fatty acid spin labels in the lipid-protein vesicles. One was immobilized presumably due to the presence of boundary lipid around the protein and the second component was indicative of anisotropic motion similar to the spectrum in phosphatidylcholine vesicles and probably due to a lamellar phase but with a slightly greater order parameter. Lipophilin was found to increase the order parameter linearly with increasing concentration of protein incorporated into the vesicles. However, the phase transition temperature as measured from the 2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPO) solubility parameter was unchanged.

INTRODUCTION

Studies of lipid-protein interactions are necessary in order to understand the organization of membranes and how this organization may be disrupted in disease states. Because biological membranes are highly complex structures consisting of many different components, such studies can be more readily interpreted when performed on well-defined model systems of pure lipids and proteins.

The present report describes the recombination of a purified, delipidated hydrophobic protein from myelin with phosphatidylcholine by solubilization of the

Abbreviations: TEMPO, 2,2,6,6-tetramethyl piperidine-1-oxyl; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid.

lipid and protein in 2-chloro-ethanol followed by dialysis against buffer. This method was used earlier by Zahler and Weibel [1] for reconstitution of erythrocyte membranes. The solvent is gradually replaced by water to allow the interaction of lipids and proteins according to the molecular forces which are operative in the native membranes.

The myelin protein used here is purified from the proteolipid of human central nervous system myelin [2] and called lipophilin. It fulfills the definition of an intrinsic membrane protein [3] having a high proportion of apolar amino acids as well as 2 % covalently bound fatty acid [4]. A recent study showed that when recombined with lipid bilayers by various methods, the protein (also called N-2) increases the Na^+ permeability, decreases the enthalpy of the gel to liquid crystal phase transition and broadens the main endothermic peak while having no effect on the midpoint of the phase transition temperature [5].

In the present study, the lipid-protein vesicles are examined by electron spin resonance spectroscopy using spin label probes incorporated into the membranes in order to obtain more detailed information about the effect of lipophilin on the molecular organization of the lipids. Evidence is presented to show that lipophilin immobilizes some of the lipid, presumably in the form of boundary lipid, while increasing the order parameter of the remaining lamellar phase.

MATERIALS AND METHODS

Preparation of lipophilin. Myelin was isolated from normal human white matter by the method of Lowden et al. [6]; lipophilin was isolated and purified by chromatography on Sephadex LH-20 in chloroform/methanol (1 : 1, v/v) containing 5 % of 0.1 M HCl by the method of Gagnon et al. [2] and stored in the lyophilized form. Preparation of the water-soluble form was by the method of Anthony and Moscarello [9].

Iodination of lipophilin. Lipophilin was iodinated with ^{125}I according to the procedure of Marchalonis [7] using lactoperoxidase. The protein was suspended in phosphate buffer, pH 7.4. After reaction for 20 min the suspension was dialysed extensively against distilled water and the final product was recovered by lyophilization and dissolved in 2-chloro-ethanol.

Lipids, spin labels, and other materials. Egg phosphatidylcholine and dipalmitoyl phosphatidylcholine were purchased from Serdary Research Laboratory (London, Ontario) and gave a single spot on thin-layer chromatography. The lipids were stored in chloroform under N_2 in sealed ampoules at −70 °C.

The fatty acid spin labels, 12-doxyl-stearic acid (II), and 16-doxyl-stearic acid (III) were obtained from Syva. TEMPO (2,2,6,6-tetramethyl piperidine-1-oxyl) (IV), 8-doxyl-palmitic acid (I), 3-doxyl-cholestane (V), and 3-doxyl-5α-androstan-17β-ol (VI) were a kind gift of J. C. Hsia, University of Toronto. Doxyl represents the 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of the parent ketone. The structures are shown in Fig. 1.

L-Histidine was purchased from Eastman Organic Chemicals and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained from Calbiochem. 2-Chloro-ethanol was from Canlab (Toronto), redistilled periodically and stored in the cold and dark.

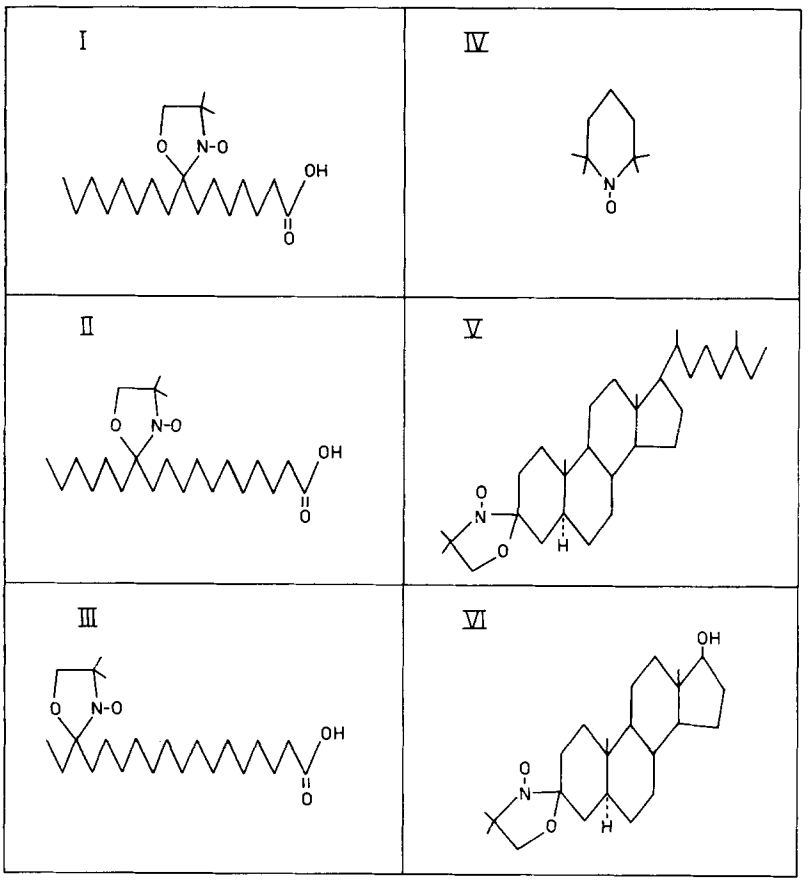

Fig. 1. Spin labels that were incorporated into lipid-protein vesicles. I, 8-doxyl-palmitic acid; II, 12-doxyl-stearic acid; III, 16-doxyl-stearic acid; IV, TEMPO; V, 3-doxyl-cholestane; VI, 3-doxyl-5α-androstan-17β-ol.

Preparation of lipid-protein vesicles. The chloroform solutions of the lipids were evaporated under N_2 and the lipid was redissolved in a small volume of 90 % 2-chloro-ethanol (usually 2 mg lipid/0.4 ml solvent). The dry protein was dissolved in 90 % 2-chloro-ethanol at a concentration of 5 mg/ml and sonicated briefly with a probe sonicator. A small aliquot of ^{125}I-labelled lipophilin containing 1.0×10^6 cpm dissolved in 2-chloro-ethanol was added to the sclution. The solution was centrifuged at 2000 rev./min for 10 min to eliminate a small amount of insoluble material. The lipid and protein solutions were combined in the desired ratios (usually 2–6 mg total weight) and were dialysed against 2 l buffer containing NaCl (10 mM), histidine (2 mM), HEPES (2 mM), and $CaCl_2$ (1 mM) or EDTA (0.1 mM) adjusted to pH 7.4. Dialysis was carried out at 4 °C for approx. 18 h with two changes of buffer. In one case, described in the text, the interaction was carried out in a temperature controlled bath at 45 °C. The buffer was saturated with nitrogen and air was excluded during the dialysis.

The resulting suspensions were centrifuged at 40 000 rev./min for 30 min at

4 °C in a SW 50.1 rotor. The supernatant was counted for ^{125}I in a Nuclear-Chicago well counter and then analyzed for phosphate content. The pellet was layered on a discontinuous sucrose gradient (10, 20, 40 and 50 % sucrose in histidine buffer) and centrifuged at 40 000 rev./min at 4 °C for 3 h. The band containing the lipid protein complex could be seen distinctly and, after removing the solution above it, was recovered with a pipet, diluted with buffer, centrifuged and washed three times to remove sucrose. The sucrose layers above and below the band were saved and counted. A pure lipid sample was always prepared in exactly the same way as a control.

The phospholipid content of the vesicle preparations was measured by phosphorus assay according to a modified Bartlett [8] procedure. Protein analysis was performed by counting ^{125}I and in some cases by amino acid analysis on a Technicon TSM amino acid analyser with good agreement between the two.

Electron microscopy. For freeze fractures, the sample was made 30 % (v/v) in glycerol and droplets of the sample (approx. 10 μl) were mounted in gold cups at 24 °C, quickly frozen in liquid Freon 22, stored no longer than 5 min in liquid nitrogen, and mounted on a specimen table of Balzer BA-360 Freeze Etch Apparatus precooled to −150 °C. Fractures were made at −115 °C at $2 \cdot 10^{-6}$ Torr, shadowed with carbon and platinum within 2 s after the last fracture and replicated with carbon. The bell jar was then filled with dry nitrogen and replicas floated on water. Replicas were cleaned in commercial sodium hypochlorite (Javex), washed twice with water and mounted on bare 75×300 mesh copper grids. All specimens were examined in a Philips EM-300 operated at 60 kV using a 50 μm objective aperture and the liquid nitrogen anticontamination device.

Electron spin resonance measurements. The vesicle preparations were resuspended in buffer at a concentration of approx. 1 mg lipid/0.1 ml buffer. A chloroform solution of the spin label was evaporated in a test tube and the lipid-protein suspensions or aqueous solutions of the protein were labelled by incubating them with the film of spin label for 30 min at room temperature. The concentration of spin label in the suspension was approx. 10^{-4} M and the mol ratio of label to lipid was approx. 1 : 100 while the ratio of label to protein was approx. 10–50 nmol per mg of protein.

Suspensions were taken up in 50-μl disposable micropipets and centrifuged at 2000 rev./min for 10 min to obtain a concentrated sample for electron spin resonance measurements. Spectra were obtained on a Varian E-4 or E-6 spectrometer with a Varian temperature control accessory which was calibrated before measurement. All measurements were made at 37 °C unless otherwise noted. The microwave power used was 10 mW.

Analysis of electron spin resonance spectra. Quantitative theories have been presented relating the parameters of the spectra of amphiphilic spin labels to molecular motion and orientation in ordered states [10–13]. This study makes use of relative changes in rotational motion, order parameter, and partition of an amphiphilic spin label into the membrane phase.

For spin labels which have fast, nearly isotropic motion, an empirical motion parameter τ_0, can be derived from spectral parameters [14]

$$\tau_0 = KW_0[(h_0/h_{-1})^{\frac{1}{2}}-1] \quad (1)$$

where $K = 6.5 \cdot 10^{-10}$ s is fixed arbitrarily at its limiting value in the case of rapid isotropic tumbling, W_0 is the width of the center line and h_0 and h_{-1} are the heights of the center and high field first derivative lines, respectively, measured as indicated in ref. 15.

The order parameter S is obtained from the anisotropic hyperfine splittings $T_{\parallel}'$ and $T_{\perp}'$ measured as shown in Fig. 4 by using the equation

$$S = \frac{(T_{\parallel}' - T_{\perp}')}{T_{zz} - T_{xx}} \tag{2}$$

where T_{zz} and T_{xx} are the rigid lattice principal hyperfine values obtained from single crystal spectra and are well documented in the literature [10]. The value used for $T_{zz} - T_{xx}$ in this study was 26 G. The order parameter S is a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chains in the lipid bilayer. Its maximum value is 1.0 for perfect order while complete disorder results in a value of $S = 0$.

In the absence of molecular motion the distance between the outer hyperfine extrema is twice the value of the maximum principal hyperfine component. As molecular motion increases, these outermost lines move in so that the distance between them, $2T_m$ measured as shown in Fig. 3, serves as a measure of the relative molecular motion.

The partitioning of the spin label TEMPO between the aqueous phase and membrane phase can be used to measure the occurrence of phase transitions. The high field peaks of the spectrum of TEMPO in the two phases can be resolved because of differences in the isotropic g and T tensors in the two environments. The height of the membrane peak H is approximately proportional to the amount of spin label dissolved in the hydrophobic phase while the height of the aqueous peak P is proportional to the amount of spin label in the polar aqueous phase where H and P were measured as shown in ref. 16. Thus the TEMPO solubility parameter f

$$f = \frac{H}{H+P} \tag{3}$$

is approximately equal to the fraction of the spin label dissolved in the membrane phase.

RESULTS

Properties of vesicles

Lipophilin was combined in various ratios with phosphatidylcholine in 2-chloro-ethanol and dialysed against buffer. Within 2 h of dialysis against buffer containing 10 mM NaCl, the solution becomes opalescent if lipophilin is present or yields a white precipitate if only phosphatidylcholine is present. The more protein present the clearer the suspension. After centrifugation of the suspension at 40 000 rev./min, 15–25 % of the initial lipid and protein remain in the supernatant with less material sedimenting at the higher protein concentrations. Sucrose density gradient centrifugation of the pellet resulted in a single sharp band located between the 10 and 50 % sucrose layers, depending on the amount of protein incorporated. For all the samples containing protein, no lipid was detected at the top and very little protein

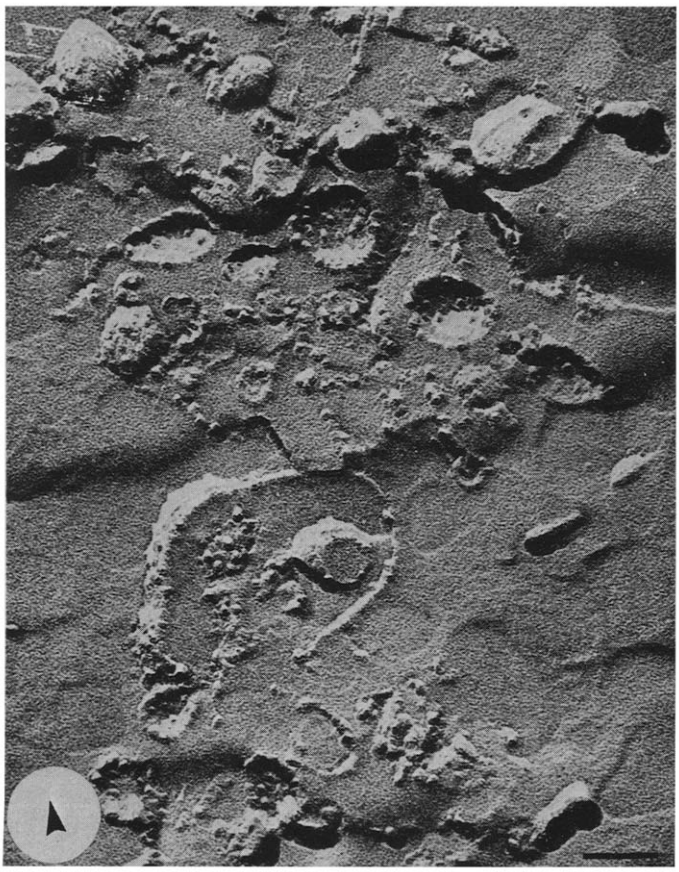

Fig. 2. Freeze fracture electron micrographs of phosphatidylcholine-lipophilin vesicles containing 40 % protein (by weight) after purification on a sucrose gradient. An arrow in the lower left-hand corner indicates the direction of the platinum deposit. Bar equivalent to 1000 Å. Total magnification is 100 000.

(as determined by ^{125}I) was found in the gradient outside the lipid-protein band. The lipid : protein ratio of the vesicles recovered from the band usually agreed quite closely with the ratio of the starting material.

The freeze fracture electron micrograph of vesicles containing 40 % lipophilin is shown in Fig. 2. The vesicles are single-layered, 1000–2000 Å in diameter, and contain numerous particles approx. 100 Å in diameter. All of the vesicles contained particles. A control sample of phosphatidylcholine prepared in the same way is multi-layered and shows completely smooth fracture surfaces.

In some experiments, lipid-protein samples were dialysed against 100 mM NaCl instead of 10 mM used above. These precipitated in the dialysis bag and much less lipid and protein remained in the supernatant after centrifugation. However, this pellet gave a more diffuse band after sucrose gradient centrifugation. Freeze fracture showed that these vesicles were multi-layered and also contained particles, while those which were dialysed against 10 mM NaCl were single-layered. The pres-

ence of up to 1 mM Ca^{2+} does not appear to have any effect on the amount of protein incorporated. Since dialysis against 10 mM NaCl gave more uniform vesicles, samples were prepared routinely in this way for electron spin resonance spectroscopy.

Dialysis of a mixture of dipalmitoylphosphatidylcholine with an initial concentration of 50 % protein at a temperature below the phase transition temperature resulted in nearly as much incorporation of protein (40 %) into dipalmitoylphosphatidylcholine as when carried out above the phase transition temperature at 45 °C (50 % incorporated). This may be due to the ab initio nature of the process since it has been shown that some water-soluble proteins are frozen out of liposomes [19] while some membrane proteins aggregate [17] below the phase transition temperature. Vesicles prepared with dipalmitoylphosphatidylcholine precipitated and were multilayered even in 10 mM NaCl.

When preparation of vesicles was attempted by evaporating a chloroform/methanol solution of the lipid and protein and dispersing in buffer, particles were seen in the fracture plane of the vesicles but large areas of aggregated protein and smooth vesicles with no protein were also present even after purification by sucrose density gradient centrifugation. Sucrose density gradient centrifugation resulted in diffuse bands also indicating non-homogeneous incorporation. Dialysis from 2-chloroethanol resulted in better incorporation of lipophilin and more homogeneous vesicles free of pure lipid vesicles and unincorporated protein. Spectra from these vesicles can therefore be interpreted more reliably.

ESR results

Boundary lipid. A series of spectra of 8-doxyl-palmitate (I) in lipid-protein vesicles with increasing protein content are shown in Fig. 3. At low protein concentrations, the spectra resemble that of pure phosphatidylcholine (Fig. 3A). As the protein content increases, a second immobilized component is clearly distinguished. At 75 % protein content, the spectrum is nearly completely immobilized (Fig.3D). However, by comparing the spectrum of the 75 % mixture to the spin label bound to the pure aqueous form of lipophilin (Fig. 3E), it can be seen that there is still a more fluid component present. Furthermore, one-half the distance between the outer hyperfine extrema in the 75 % mixture, $T_m = 30.1$ G while for the spin label bound to aqueous lipophilin $T_m = 32.4$ G. Thus the spin label in the lipid-protein vesicles is not quite as immobilized as when bound directly to the protein. A small difference in T_m could also be due to a difference in polarity experienced by the spin label. Nevertheless, this difference indicates that the spin label immobilized in the lipid protein vesicles experiences a slightly different environment from that bound directly to the protein. A similar difference is found for the probes 12-doxyl-stearate (II), 16-doxyl-stearate (III), and androstan spin label (VI) (Table I).

This immobilization of the spin label in the lipid-protein vesicles must be due to the presence of immobilized lipid surrounding the protein similar to the boundary lipid postulated for cytochrome oxidase [18] and cytochrome b_5 [20]. The immobilized component is undoubtedly present at the lower protein concentrations but is obscured by the high intensity of the fluid component. Similar spectra were also obtained for 16-doxyl-stearate with better resolution between the immobilized and fluid components since 16-doxyl-stearate has nearly isotropic motion in phosphatidylcholine. The T_m values (Table I) for all the fatty acid labels in the immobilized phase

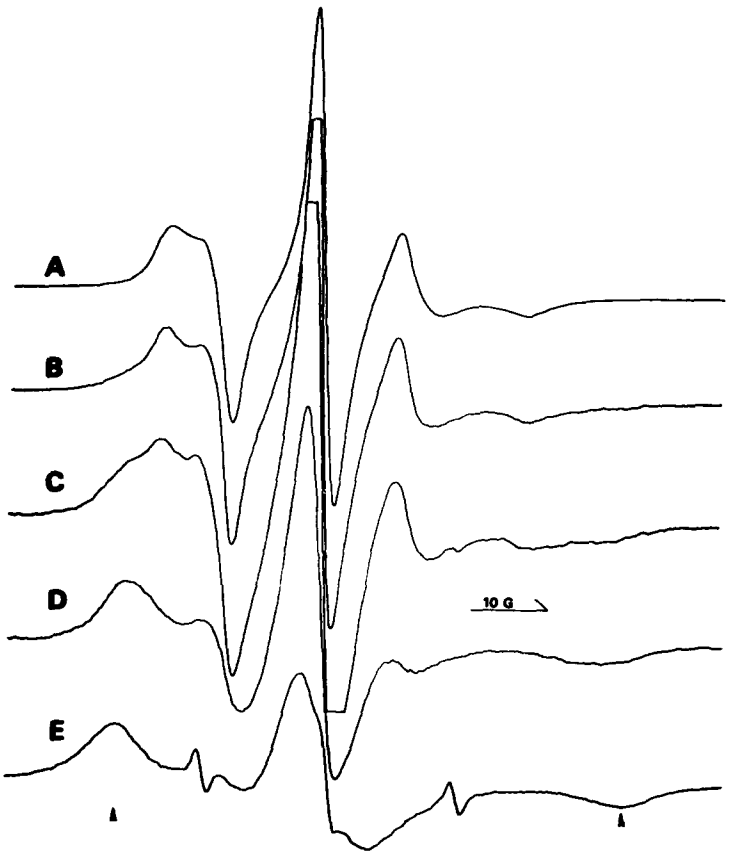

Fig. 3. Electron spin resonance spectra of 8-doxyl-palmitate in aqueous suspensions of phosphatidylcholine with varying lipophilin content. A, phosphatidylcholine only; B, phosphatidylcholine with 18 % lipophilin; C, phosphatidylcholine with 40 % lipophilin; D, phosphatidylcholine with 75 % lipophilin; E, aqueous lipophilin only. Lipophilin content is percentage of total weight. $2T_m$ is measured as the separation of the outermost lines indicated by the arrows.

TABLE I

MAXIMUM HYPERFINE SPLITTING (T_m IN G) OF PROBES BOUND TO AQUEOUS LIPOPHILIN AND PHOSPHATIDYLCHOLINE-LIPOPHILIN VESICLES

	I	II	III	VI
Aqueous lipophilin	32.4	33.0	32.6	33.3
Phosphatidylcholine-lipophilin vesicles	30.1	31.6	31.3	31.9

are quite similar which indicates that this phase has no fluidity gradient as seen in a lamellar [12, 21] or hexagonal [22] fluid lipid phase. The immobilized phase does not appear to have a polarity gradient which occurs in lipid bilayers even when frozen [20] although at the temperature used here (37 °C), the T_m values for the fatty acids could also be affected by differences in immobilization.

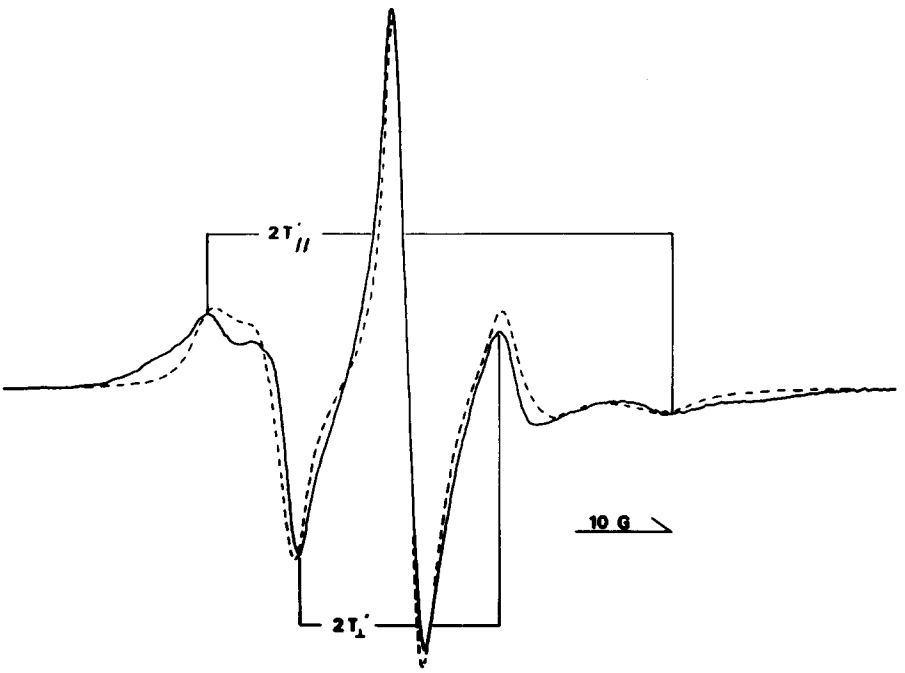

Fig. 4. Electron spin resonance spectra of 8-doxyl-palmitate in aqueous suspensions of phosphatidylcholine (– – –) and phosphatidylcholine with 34 % lipophilin (—). $2T_{\parallel}'$ and $2T_{\perp}'$ are measured as indicated.

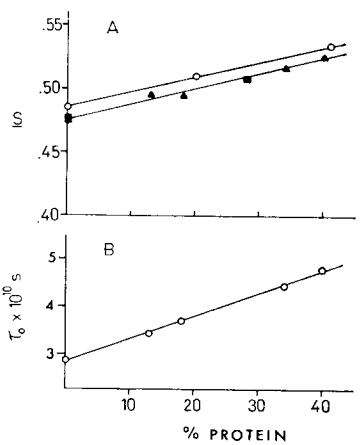

Fig. 5. (A) Dependence of the order parameter S of 8-doxyl-palmitate on lipophilin content in phosphatidylcholine vesicles. Concentration of lipophilin is expressed as percentage of total weight. The symbols ▲, ○, ■, represent values obtained in three separate experiments. (B) Dependence of the motion parameter τ_0 of 16-doxyl-stearate on lipophilin content in phosphatidylcholine vesicles. Data is from a single experiment.

Increase in order of fluid phase

At protein concentrations where the immobilized component does not contribute significantly to the spectrum, the effect of lipophilin on the order parameter of 8-doxyl-palmitate and mobility of 16-doxyl-stearate can be measured. In Fig. 4 the spectrum of 8-doxyl-palmitate in vesicles containing 34 % lipophilin is compared with that of pure phosphatidylcholine which has been treated in exactly the same manner. The order parameter for pure lipid vesicles obtained by dialysis is identical to that in vesicles prepared by dispersing a lipid film in buffer, which are known to have bilayer structure. In the lipid-protein vesicles, the line shape of the fluid component is similar indicating anisotropic motion of the spin label is retained. However, the order parameter is greater. Fig. 5 shows the dependence of the order parameter S of 8-doxyl-palmitate and the motion parameter τ_0 of 16-doxyl-stearate on the percentage of lipophilin in the vesicles and indicates greater order both near the surface and within the interior of the bilayer with increasing protein content. The motion parameter of the rigid steroid androstan spin label (VI) was also greater in vesicles containing lipophilin (Table II).

TABLE II

MOTION PARAMETER OF PROBES IN PHOSPHATIDYLCHOLINE VESICLES WITH OR WITHOUT 40 % LIPOPHILIN AT 37 °C

	$\tau_0(\times 10^{10})$ s	
	16-doxyl-stearate (III)	Androstan (VI)
Phosphatidylcholine vesicles	2.9	5.2
Phosphatidylcholine-lipophilin vesicles	4.7	6.5

Effect of lipophilin on phase transition

The effect of lipophilin on the phase transition of dipalmitoylphosphatidylcholine was measured from the TEMPO spectral parameter f. Fig. 6 shows the phase transition of pure dipalmitoylphosphatidylcholine vesicles compared with that in vesicles containing 50 % protein. The presence of protein does not alter the mid-point of the phase transition temperature but abolishes the premelt transition in agreement with differential scanning calorimetry [5]. However, it can be seen that in the presence of lipophilin a greater percentage of the bound TEMPO binds after the transition is complete than in the case of pure dipalmitoylphosphatidylcholine. This indicates that either the partition coefficient of TEMPO in the boundary lipid increases with temperature or that some of the boundary lipid is converted to fluid bilayer lipid at higher temperatures allowing increased binding of TEMPO after the phase transition has occurred. Although the amount of boundary lipid does appear to decrease with temperature, a substantial proportion of the total lipid is still immobilized at 50 °C. Both the structure and the amount of the boundary lipid are probably temperature dependent.

The temperature dependence of the mobility of 16-doxyl-stearate in phosphatidylcholine vesicles containing 18 % lipophilin is displayed on an Arrhenius plot in Fig. 7. Although the motion parameter τ_0 is greater for the preparation containing

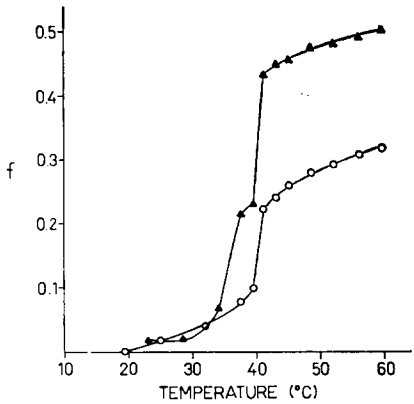

Fig. 6. TEMPO solubility parameter f plotted as a function of temperature for vesicles of pure dipalmitoylphosphatidylcholine (▲) and vesicles of dipalmitoylphosphatidylcholine containing 50 % lipophilin (○). Less material is present in the sample tube in the case of vesicles containing lipophilin.

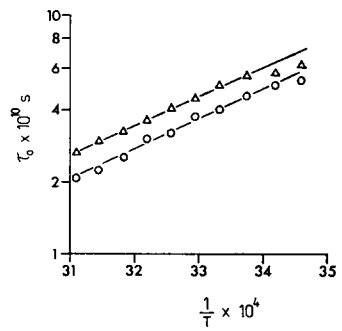

Fig. 7. Arrhenius plot of the motion parameter τ_0 of 16-doxyl-stearate in vesicles of pure phosphatidylcholine (○) and phosphatidylcholine containing 18 % lipophilin (△).

protein indicating decreased mobility, the slope is similar indicating that the activation energy for the fatty acid motion is not significantly different from that in pure phosphatidylcholine vesicles. Thus, although lipophilin decreases the fluidity of the lamellar phase, it must do so without significantly altering the energy of the interaction between the membrane components.

Location of steroid probes

Evidence has been reported to indicate that cholesterol may be excluded from the lipid surrounding the Ca^{2+}-ATPase from sarcoplasmic reticulum [24]. However, the proteolipid from myelin has been reported to have a strong interaction with cholesterol monolayers [25]. The location of cholesterol in the phosphatidylcholine-lipophilin vesicles was studied by monitoring the location of spin label steroids, 3-doxyl-cholestane (V) and androstan spin label (VI) in the vesicles as models for cholesterol. Even in the presence of 40 % protein, there is very little immobilized component in the spectrum as shown in Fig. 8A for the androstan spin label. The

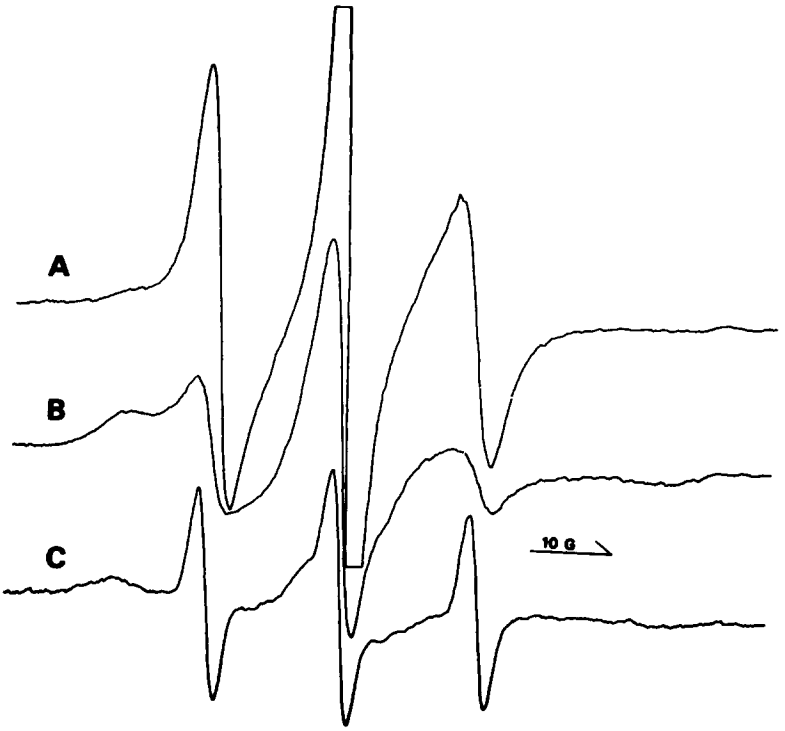

Fig. 8. Electron spin resonance spectra of androstan spin label (VI) in (A) phosphatidylcholine-lipophilin vesicles containing 40 % protein, (B) phosphatidylcholine-lipophilin vesicles containing 75 % protein, and (C) aqueous solution of lipophilin only. The sharp lines in C are due to the label in the aqueous phase. In C, lipophilin and spin label are present in the same concentration as in Fig. 3E. However, C above is recorded at an instrument sensitivity five times greater than that used in Fig. 3E.

spectrum in Fig. 8A is expanded in order to show the low intensity immobilized peak. The ratio of the immobilized to fluid peak heights cannot be compared for the fatty acid probe and the steroid probe since the motion is different; however, it is clear that a much smaller proportion of the total signal is immobilized for the androstan spin label than for 8-doxyl-palmitate in Fig. 3C. The binding of this probe to the aqueous form of the protein is much less than the fatty acid probes (Fig. 8C). However, there is a significant immobilized component in the spectrum of vesicles containing 75 % protein (Fig. 8B). Thus, some steroid is immobilized by the boundary layer but apparently not as much as the fatty acids.

DISCUSSION

Dialysis from 2-chloro-ethanol resulted in the homogeneous incorporation of lipophilin into lipid vesicles as judged by centrifugation on a sucrose density gradient and freeze fracture microscopy and may prove to be a useful method for reconstitution of membranes. Since lipophilin has no known biological activity, it is difficult to assess whether or not the native structure is retained by this treatment. However, lipophilin has a high percentage of α-helical structure in chloroform/methanol and in

2-chloro-ethanol and this is retained upon incorporation into lipid vesicles (Epand, R., Boggs, J. M. and Moscarello, M. A., unpublished).

When incorporated into lipid vesicles lipophilin immobilized part of the lipid and increased the order of the remaining fluid lamellar phase. X-ray diffraction studies indicate the presence of well-ordered lipid lamellae upon interaction of lipophilin with phosphatidylcholine [23]. The freeze fracture results indicate that the protein is incorporated into the hydrocarbon region of the bilayer and it probably spans at least one-half of the bilayer since it increases the order throughout the bilayer. Since the activation energy for the motion of the fatty acid chains is similar in the presence of lipophilin, the protein probably increases the order through long range effects on packing similar to the effect of cholesterol. This explains why there is no change in the temperature of the mid-point of the phase transition. An increase in order due to incorporation of a hydrophobic protein has also been found for rhodopsin [26] and a proteolipid from sarcoplasmic reticulum [27]. No immobilized lipid was detected for the latter two proteins but the lipid : protein ratio was higher than that used in the present study. The immobilized lipid seen here is similar to the boundary lipid observed in the presence of cytochrome oxidase [18] and cytochrome b_5 [20].

It has been shown that lipophilin decreases the enthalpy of the phase transition [5] indicating that it prevents some of the lipid, approximately half at 50 % lipophilin content, from participating in the phase transition. The present study shows that some of the lipid does not go through the phase transition because it has been immobilized by the protein.

The structure of the boundary lipid is not known but it is not an anisotropic phase such as lamellar or hexagonal phases since it does not possess a fluidity or polarity gradient. Although it does not undergo a phase transition, its structure and amount may change with temperature since TEMPO continues to bind to lipid-protein vesicles after the lamellar phase has already gone through its phase transition. The boundary lipid probably does not have an ordered lattice structure but rather conforms to the irregular surface of the protein. It is significant that the rigid steroids do not bind as well to the boundary layer or surface of the protein as the flexible fatty acids.

The important question is whether these effects of lipophilin on lipid organization are relevant to its effects in intact myelin. Myelin has greater order than its total lipid extract and the present study shows that lipophilin probably contributes to this greater order (Boggs, J. M. and Moscarello, M. A., unpublished). Since proteolipid isolated from myelin contains tightly bound lipid which cannot even be extracted from it with chloroform/methanol [28], the interaction between the aproprotein and immobilized boundary lipid probably exists in myelin also. Incorporation of lipophilin into lipid vesicles results in particles which can be seen on the freeze fracture faces. Similar particles which have been observed on the fracture faces of frozen myelin, may thus be due to the proteolipid in myelin (ref. 29 and Vail, W. J. and Moscarello, M. A., unpublished).

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