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Effect of Basic Protein from Human Central Nervous System Myelin on Lipid Bilayer Structure

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Summary. The effect of myelin basic protein from normal human central nervous system on lipid organization has been investigated by studying model membranes containing the protein by differential scanning calorimetry or electron spin resonance spectroscopy. Basic protein was found to decrease the phase transition temperature of dipalmitoyl phosphatidylglycerol, phosphatidic acid, and phosphatidylserine. The protein had a greater effect on the freezing temperature, measured from the cooling scan, than on the melting temperature, measured from the heating scan. These results are consistent with partial penetration of parts of the protein into the hydrocarbon region of the bilayer in the liquid crystalline state and partial freezing out when the lipid has been cooled below its phase transition temperature.

The effect of the protein on fatty acid chain packing was investigated by using a series of fatty acid spin labels with the nitroxide group located at different positions along the chain. If the protein has not yet penetrated, it increases the order throughout the bilayer in the gel phase, probably by decreasing the repulsion between the lipid polar head groups. Above the phase transition temperature, when parts of it are able to penetrate, it decreases the motion of the lipid fatty acid chains greatly near the polar head group region, but has little or no effect near the interior of the bilayer. Upon cooling again the protein still decreases the motion near the polar head group region but increases it greatly in the interior. Thus, the protein penetrates partway into the bilayer, distorts the packing of the lipid fatty acid chains, and prevents recrystallization, thus decreasing the phase transition temperature.

The magnitude of the effect varied with the lipid and was greatest for phosphatidic acid and phosphatidylglycerol. It could be reversed upon cooling for phosphatidylglycerol but not phosphatidic acid. The protein was only observed to decrease the phase transition temperature of phosphatidylserine upon cooling. It had only a small effect on phosphatidylethanolamine and no effect on phosphatidylcholine. Thus, the protein may penetrate to a different extent into different lipids even if it binds to the polar head group region by electrostatic interactions.

Myelin has a relatively simple composition with a low protein content, and yet its major proteins include both an intrinsic and an extrinsic type of membrane protein which makes it a useful membrane for studying the effects of both types of proteins on membrane structure. Both of the major proteins, the proteolipid which is of the intrinsic type and the basic protein

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or encephalitogenic protein which seems to be of the extrinsic type, have been purified and can be incorporated into lipid vesicles for study of their individual properties. Several studies on model membranes containing a hydrophobic protein fraction called lipophilin, purified from the proteolipid of human central nervous system (CNS) myelin (Vail, Papahadjopoulos & Moscarello, 1974; Papahadjopoulos *et al.*, 1975*b*; Boggs, Vail & Moscarello, 1976; D.D. Wood, J.M. Boggs & M.A. Moscarello, *unpublished results*), have shown that this protein is embedded in the hydrocarbon region, probably spanning the bilayer and forming intramembranous particles which can be seen by freeze fracture. The protein immobilizes some lipid around it in the form of boundary lipid, similar to that of other intrinsic membrane proteins (Jost *et al.*, 1973; Dehlinger, Jost & Griffith, 1974), thus decreasing the enthalpy of the phase transition. It has a small ordering effect on the remaining lipid without altering its phase transition temperature.

The basic protein of myelin induces the autoimmune demyelinating disease, experimental allergic encephalomyelitis (EAE), when injected into animals. It appears to interact with lipid primarily through electrostatic interactions since it binds only to acid lipids (Palmer & Dawson, 1969; Demel *et al.*, 1973; Steck *et al.*, 1976), has 25% basic amino acids distributed throughout the molecule and its binding can be inhibited by high salt concentrations. However, studies with bovine basic protein indicate that part of the molecule penetrates into the hydrocarbon region to interact hydrophobically with the lipid fatty acid chains, since it increases the permeability of lipid vesicles to glucose (Gould & London, 1972) and Na^+ (Papahadjopoulos *et al.*, 1975*a*) and expands monolayers (Demel *et al.*, 1973; Papahadjopoulos, 1975). It decreases the enthalpy and temperature of the lipid phase transition, indicating that it disrupts the hydrophobic interactions between the lipid fatty acid chains and reduces the size of the lipid cooperative unit which undergoes the transition (Papahadjopoulos *et al.*, 1975*a*). Furthermore, when the protein is bound to lipid, certain parts of the molecule are protected from hydrolysis by trypsin and other enzymes (London & Vossenburg, 1973; London *et al.*, 1973), indicating that these regions are buried in the lipid bilayer.

Although basic protein seems to penetrate into the hydrocarbon region, its effects on the molecular organization of the fatty acid chains have not yet been elucidated. It was suggested, on the basis of the above effects, that basic protein may fluidize the bilayer (Papahadjopoulos *et al.*, 1975*a*). However, the protein appears to interact only electrostatically with the lipid bilayer below its phase transition temperature. It can penetrate to its full extent,

resulting in a decrease in the phase transition temperature (T_c), only when the lipid is in its liquid crystalline phase and indeed if the lipid is cooled below the transition temperature again, the protein is eventually partially frozen out (Papahadjopoulos *et al.*, 1975*a*).

In the present study, lipid-protein vesicles containing human CNS basic protein are examined by electron spin resonance spectroscopy (ESR), using spin label fatty acid probes with the nitroxide group at varying positions along the chain in order to obtain more detailed information about the effect of this protein on lipid fatty acid chain packing above and below the phase transition temperature. Its effect on lipid organization varied with different lipids.

Materials and Methods

Preparation of Basic Protein

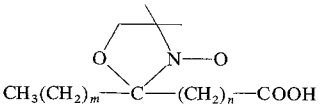
Myelin was isolated from normal human white matter and basic protein extracted from it by the method of Lowden, Moscarello & Morecki, (1966). The protein was stored in the lyophilized form. For some experiments, basic protein was iodinated with ^{125}I according to the procedure of Marchalonis (1969) using lactoperoxidase. An aliquot was added to the basic protein solution.

Lipid, Spin Labels, and Other Materials

Egg phosphatidylethanolamine (PE) was a gift from Dr. W.J. Vail, University of Guelph, and bovine brain phosphatidylserine (PS), egg phosphatidic acid (PA), egg phosphatidylcholine (PC), dipalmitoyl phosphatidic acid (DMPA) and dipalmitoyl phosphatidylglycerol (DPPG) were gifts from Dr. D. Papahadjopoulos, Roswell Park Memorial Institute, Buffalo. The lipids were chromatographically pure and were stored in chloroform under N_2 in sealed ampules at $-70\,°C$.

The fatty acid spin labels, 5-doxyl-stearic acid I (12,3), 12-doxylstearic acid I (5,10) and 16-doxyl-stearic acid I (1,14) were obtained from Syva; 8-doxyl-palmitic acid I (7,6) was a gift from Dr. J.C. Hsia, University of Toronto. Doxyl represents the 4′, 4′-dimethyl-oxazolidine-N-oxyl derivative of the parent ketone.

L-histidine was purchased from Eastman Organic Chemicals and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) was obtained from Calbiochem; 2-chloroethanol was purchased from Coleman and Bell, redistilled periodically and stored at 4 °C in the dark.



$CH_3(CH_2)_m—C—(CH_2)_n—COOH$ I(m, n)

Preparation of Lipid-Protein Vesicles

The vesicles were prepared by three different methods: (i) the chloroform solutions of the lipids were evaporated under N_2 and to the dry film was added buffer containing the desired

amount of basic protein which had been dissolved and centrifuged to remove any insoluble material. The material was suspended by vortex shaking for 10 min at room temperature or at 45° in the case of DPPG, and 57° in the case of DMPA; (ii) the lipid was suspended in buffer without basic protein and the suspension was sonicated in a bath-type sonicator under N_2 at room temperature until opalescent and the desired amount of basic protein dissolved in the buffer was added. It was not determined whether single-layered vesicles were obtained after sonication, and in the case of PE, in particular, they may have been multilayered. Sonication was performed mainly to facilitate dispersion into smaller lipid vesicles before interaction with the protein; (iii) the chloroform solutions of the lipids were evaporated under N_2 and redissolved in 100% 2-chloroethanol. The basic protein was dissolved in 100% 2-chloroethanol with sonication and the desired amount added to the lipid. The 2-chloroethanol solutions of lipid and protein were placed in dialysis tubing (3,500 mol wt cutoff) which had been boiled in EDTA, and were dialyzed against 2 liters of buffer at room temperature for approximately 18 hr with two changes of buffer. The buffer was saturated with nitrogen, and air was excluded during the dialysis. The buffer used contained NaCl (10 mM), HEPES (2 mM), and EDTA (1 mM), adjusted to pH 7.4.

Precipitates were always obtained in the presence of basic protein and were centrifuged at 10,000 × *g* at room temperature for 5 min, and the pellets were used for calorimetry or spin labeling. The lipid vesicles prepared by dialysis from 2-chloroethanol usually had to be collected by centrifugation at 40,000 rpm at 4 °C for 1 hr in a SW 50.1 rotor. When the lipid-protein vesicles were prepared by using sonicated liposomes the lipid control was prepared by dispersion. If ^{125}I-labelled protein was used, the supernatant was counted for ^{125}I in a Nuclear-Chicago well counter and analyzed for phosphorus by a modified Bartlett (1959) procedure. The lipid protein vesicles were initially purified by centrifugation on a discontinuous sucrose gradient (10, 20, 40 and 50% sucrose in buffer) and centrifuged at 40,000 rpm for 3 hr at 4 °C. However, very little uncombined lipid and protein was found on the gradient so this step was later omitted. The lipid protein ratio of the vesicles was measured by determining lipid content by phosporus assay and protein content by amino acid analysis on a Technicon TSM amino acid analyzer, after hydrolysis with 5.7 N HCl for 19 hr at 110°.

Differential Scanning Calorimetry

Samples were run on a Perkin-Elmer DSC-2 usually at a heating or cooling rate of 5° or 10°/min. However, since significant hysteresis occurs at these rates for all samples including the pure lipids, samples of DPPG, with and without basic protein, were also run at the slowest rate possible on this instrument, 0.31°/min. Identical effects of basic protein were seen at these slow rates as at the higher rates. The midpoint of each peak was defined as the phase transition temperature (T_c). The enthalpy of the transition was not determined.

Electron Spin Resonance Measurements

A chloroform solution of the spin label was evaporated in a test tube, and the vesicles were labeled by incubating them with the film of spin label for 30 min at room temperature. The concentration of spin label in the suspension was approximately 10^{-4} M, and the mole ratio of label to lipid was approximately 1:100. Suspensions were taken up in 50 µl disposable micropipettes and centrifuged at 2,000 rpm for 10 min to obtain a concentrated sample for electron spin resonance measurements. Spectra were obtained on a Varian E-4 spectrometer with a Varian temperature control accessory. All measurements were made at 37° unless otherwise noted. The microwave power used was 10 mW.

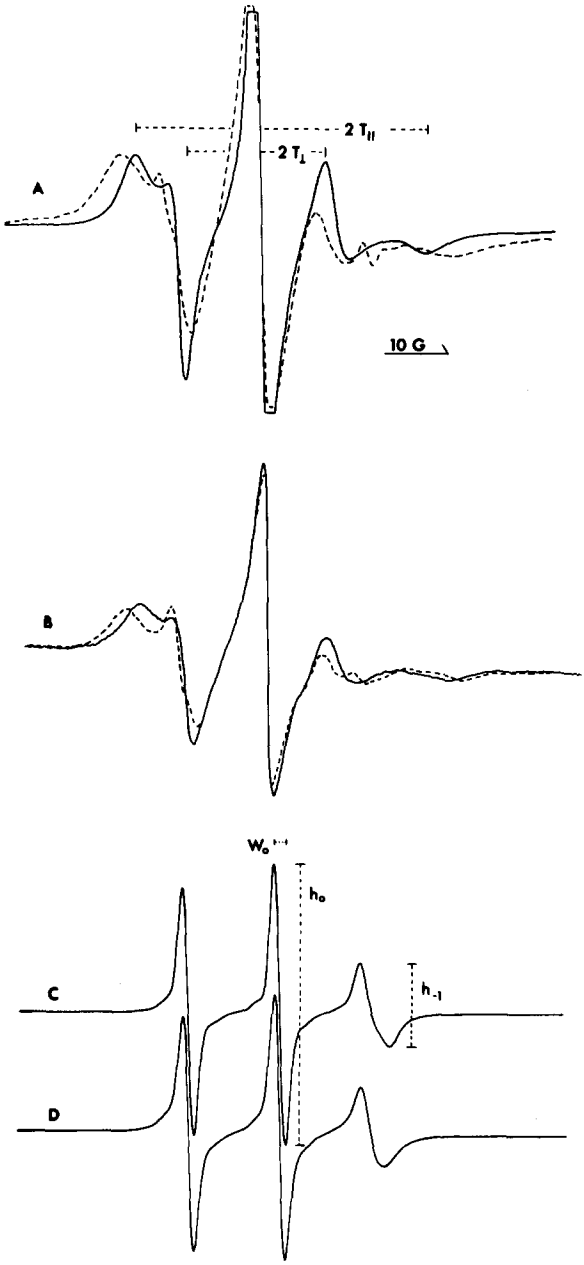


Fig. 1. Electron spin resonance spectra at 37° of: (*A*): 5-doxyl-stearate in vesicles of PA (——) and PA + 40% basic protein (------), $2T_{11}$ and $2T_{\perp}$ are measured as indicated; and (*B*): Vesicles of PE (——) and PE + 30% basic protein (------); (*C*): 16-doxyl-stearate in vesicles of PA; and (*D*): 16-doxyl-stearate in vesicles of PA + 40% basic protein. W_0, h_0 and h_{-1} are measured as shown. The isotropic spectral component seen in the spectra of vesicles containing protein is probably due to spin label in the aqueous phase and is apparent for these samples because the ratio of pellet size to supernatant is less than for samples of lipid

Analysis of Electron Spin Resonance Spectra

This study makes use of relative changes in rotational motion and order parameter of fatty acid spin labels. For spin labels such as I (1,14) which have fast, nearly isotropic motion, an empirical motion parameter τ_0 can be derived from spectral parameters (Eletr & Keith, 1972),

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

where $K = 6.5 \times 10^{-10}$ sec 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 Fig. 1.

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. It is obtained from the anisotropic hyperfine splittings T_{11} and $T_\perp$ measured as shown in Fig. 4 by using the equation (Seelig, 1970; Hubbell & McConnell, 1971).

$$S = \frac{T_{11} - T_\perp}{T_{zz} - T_{xx}} \quad (2)$$

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 (Seelig, 1970). The maximum value of S is 1.0 for perfect order, while complete disorder results in a value of $S = 0$.

Results

Preparation of Vesicles

Addition of basic protein to preformed sonicated vesicles resulted in precipitation of the vesicles. When the procedure of dialysis was used to prepare the vesicles, a precipitate was obtained in the presence of the basic protein but not for any of the acidic lipids used here (phosphatidylcholine, however, precipitates). It was therefore necessary to centrifuge the lipid at 100,000 × g in order to sediment a small fraction of it while at least 75 % of the lipid could be sedimented in the presence of basic protein. However, PS vesicles could not be sedimented to a significant extent when concentrations of basic protein less than 30 % were used.

As shown in Table 1, similar amounts of protein were incorporated regardless of the method used to prepare the vesicles. However, the different lipids bound varying amounts of protein. PE was saturated at approximately 29 %, while PS could bind up to 87 %. However, much of this binding to PS may represent nonspecific binding due to aggregation of PS vesicles. The maximum amount found in DPPG was 50 %. Binding was not investigated in detail for PA, but at concentrations up to 40 % it was found to bind all of the protein present. Basic protein did not interact with PC vesicles; the amount bound to PC prepared by any method was insignificant.

Table 1. Amount of basic protein incorporated into lipid vesicles composed of different lipids and prepared by different methods

Lipid	Method of preparation	Initial concentration of basic protein (% w/w)	Concentration of basic protein incorporated (% w/w)
PE	Sonicated preformed vesicles	10	11.8
		20	19.6
		40	28.8
		60	27.4
PE	Dialysis	10	10
		20	13
		40	26
		60	29
PS	Sonicated preformed vesicles	10	9.8
		24	27.6
		40	51.7
		60	70
PS	Dialysis	10	3
		20	14
		40	55
		60	87
PA	Dispersed	30	34
		40	40
PA	Dialyzed	20	21.4
DMPA	Dispersed	30	20
DPPG	Dispersed	30	28
		50	40
		65	50
DPPG	Dialyzed	50	38
DPPG	Sonicated preformed vesicles	50	43

All of these lipids form a lamellar phase in the absence of basic protein (Papahadjopoulos & Miller, 1967). Although PE cannot be sonicated to form single layered vesicles at neutral pH, X-ray diffraction shows that hydrated films of this lipid are lamellar (Rand, Tinker & Fast, 1971). These films can be dispersed to give multilayered vesicles which show birefringence characteristic of the lamellar phase when examined under a polarizing microscope (Boggs & Hsia, 1973). Freeze-fracture electron microscopy also showed that PE prepared by dialysis from 2-chloroethanol formed large spherical vesicles which were probably multilayered (J.G.

Stollery & W.J. Vail, *unpublished observations*). Unilamellar vesicles of PE can be prepared by sonication at high pH followed by dialysis against buffer at neutral pH (Stollery, 1977). Freeze-fracture studies showed that addition of basic protein to these unilamellar vesicles resulted in fusion into larger multilamellar aggregates. In freeze-fracture studies of basic protein-lipid complexes formed by various methods, no hexagonal structures were ever detected (W.J. Vail, *unpublished results*). Therefore, all of the lipid dispersions and lipid-protein complexes studied here consist of multilayered aggregates of sufficiently large size that the radius of curvature of the vesicles is not important.

Similar results were obtained by ESR and DSC for vesicles prepared by any of the methods. However, the magnitude of the effect was sometimes a little less for vesicles prepared by dispersion.

Effect of Basic Protein on ESR Spectrum

The effects of basic protein on the ESR spectra of 5-doxyl-stearate in PA and PE are shown in Fig. 1 *A* and *B*, respectively. The spectra in the presence of basic protein are still characteristic of anisotropic motion of the spin label. However, basic protein induces considerable broadening of the spectrum for PA but not PE (Fig. 1 *B*). The changes in hyperfine splitting indicate that the order parameter is greatly increased in both PA and PE, indicating that the fluctuation of the spin label about an average orientation decreases. However, the broadness of the spectrum in PA indicates that there is a considerable increase in the width of the distribution of orientations of the spin label. The protein also broadened the spectrum and increased the order parameter of DPPG (at 50 °C) and PS. The effect of increasing concentration of basic protein on the order parameters of 5-doxyl-stearate or 8-doxyl-palmitate in PA, PE, DPPG and PS are shown in Fig. 2. The greatest effect occurred for DPPG, followed by PS and PA, with only a small effect on PE. Thus, basic protein bound to PA, PG and PS disorders the lipid fatty acid chains near the polar head group region while decreasing their mobility and freedom of motion, whereas in PE it increases the order by decreasing the fluctuation of the fatty acid chains.

There is no evidence for an immobilized component in the spectrum of either 5-doxyl-stearate (Fig. 1 *A* and *B*) or 16-doxyl-stearate (Fig. 1 *D*) in basic protein-lipid vesicles as was seen for lipophilin-phospholipid vesicles (Boggs *et al.*, 1976). Furthermore, basic protein in solution (1 mg/ml) does not bind or immobilize any significant amount of spin label while the

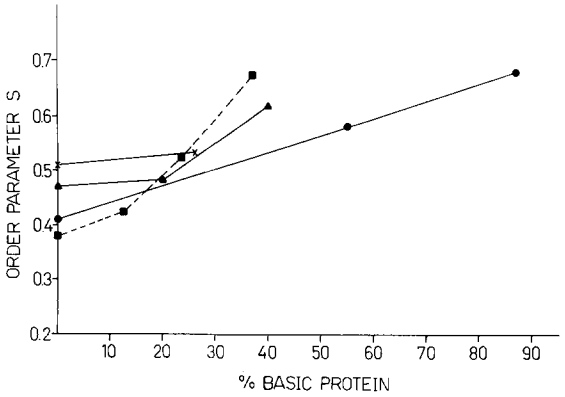


Fig. 2. Dependence of the order parameter S of 5-doxyl-stearate on concentration of basic protein (% by weight) incorporated in vesicles of PA (▲) and DPPG (■) and 8-doxyl-palmitate in vesicles of PE (×) and PS (●). All spectra were measured at 37° except for DPPG, which was at 50°

aqueous form of lipophilin at the same concentration did (Boggs *et al.*, 1976). Therefore, basic protein does not appear to immobilize lipid in the form of boundary lipid as intrinsic membrane proteins do.

Effect on Phase Transition Temperature

As reported earlier (Papahadjopoulos *et al.*, 1975*a*), incorporation of basic protein into dipalmitoyl phosphatidylglycerol vesicles induces a large decrease in the phase transition temperature. However, only the transition temperature on melting was determined and an initial concentration of 65% (which resulted in 50% incorporated) was required before an appreciable effect was seen. The heating and cooling curves for DPPG vesicles containing 28% and 50% basic protein are shown in Fig. 3. Although at 28% protein content only a small decrease (1 °C) in T_c is seen upon heating, the protein produces a decrease in T_c of 4° upon cooling, with another component freezing at even lower temperatures (Table 2). The heating curve for DPPG containing 50% protein is similar to that reported by Papahadjopoulos *et al.*, (1975*a*) and shows two transitions, one at 4.7° lower and the other 11.6° lower than the pure lipid. On cooling, a single broad peak is observed which is 12.5° lower than that of the pure lipid. The decrease seen for the heating curve is often not as great the first time the lipid is melted as on subsequent heating scans, even though the vesicles are prepared at a temperature above T_c. However, the effects are reproducible upon several heating and cooling cycles after the lipid has been melted once.

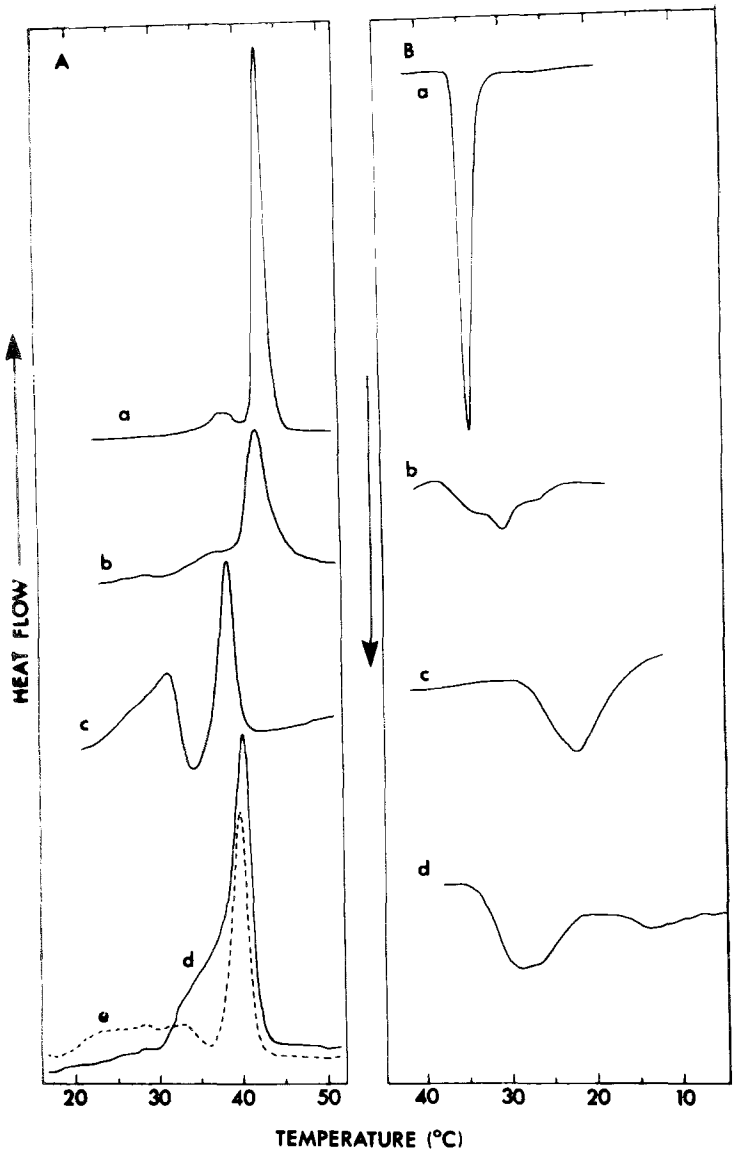


Fig. 3. Differential scanning calorimetry thermograms for DPPG with and without basic protein. (*A*): Heating scans for (*a*) DPPG; (*b*) DPPG+28% basic protein prepared by dispersion at 45°; (*c*) DPPG+50% basic protein prepared by dispersion at 45°; (*d*) first heating scan for DPPG+43% basic protein (——) prepared by adding basic protein in solution to preformed sonicated vesicles at 4 °C; (*e*) second heating scan for vesicles in (*d*); (------), same instrument settings as in (*d*). Heating rate 10°/min. (*B*): Cooling scans for (*a*) DPPG; (*b*) DPPG+28% basic protein prepared by dispersion at 45°; (*c*) DPPG+50% basic protein prepared by dispersion at 45°; (*d*) DPPG+43% basic protein prepared by adding basic protein in solution to preformed sonicated vesicles at 4 °C. Repeated cooling scans gave similar results. Cooling rate 10°/min. Similar results were observed for vesicles containing basic protein at heating and cooling rates of 0.31°/min. At this heating rate, transition temperatures for the pure lipid on heating and cooling are nearly identical

Table 2. The effect of basic protein on phase transition temperatures of various lipids from heating and cooling curves

Lipid[a]-protein vesicles	T_c heating[b]	T_c cooling[b]
DPPG dispersed at 45°	42.4	34.7
+28% basic protein	28/36/41.3	27/30.8/33.4
+50% basic protein	30.8/37.7	22.2
DPPG+38% basic protein dialysed at 45°	26.8/33.2/40.1	14/26 to 29
DPPG sonicated liposomes +43% basic protein added at 4 °C	*1st scan* 40.2 *2nd scan* 23.2/32.8/40.1	13.7/26.5 to 30.3
PA	17	7
+34% basic protein	9/17	−3.3 to −0.7/7
+50% basic protein	3.7/8	−8.5/4.9
DMPA	52.6	43.4
+20% basic protein	48/53.3	38.9/44.3
PS	5.3	0.3/3[c]
+30% basic protein	5.0	−4.1/−1[c]
+50% basic protein	–	−11.7[c]
PE	10.3	6.3[c]
+25% basic protein	9.1	3.7/4.8[c]

[a] Protein concentrations expressed as percent bound by weight determined by lipid, protein analysis on pellets.
[b] Both heating and cooling runs were performed at 10°/min except where noted. Midpoint temperatures of main transitions observed are shown. Largest peaks are underlined.
[c] Recorded at 5°/min.

Papahadjopoulos *et al.* (1975*a*) also showed that, if the vesicles were prepared at a temperature below T_c, the basic protein binds to the vesicles aggregating them but does not decrease T_c significantly. However, after brief exposure to a temperature above T_c, the large decrease in transition temperature described above occurs. It was suggested that the decrease in transition temperature is caused by partial penetration of the protein into the hydrocarbon region when the lipid is in the liquid crystalline phase. This effect could be partially reversed by incubation at 0° overnight, indicating that the protein is partially frozen out.

A similar experiment, where 43% basic protein is bound to preformed sonicated vesicles at 4 °C, is shown in Fig. 3. A small effect, a decrease in T_c of 2.2°, was seen for the first heating scan (Fig. 3*d*). For the second heating scan (Fig. 3*e*), the main endothermic peak is still decreased about 2°, but another

component 9.6° or more below that of pure DPPG is present, indicating that partial penetration of the protein has occurred. Upon cooling, however, the major peak is decreased even further, by 6.1° with a component at an even lower temperature. Thus, the results presented in Fig. 3 show that basic protein decreases the freezing temperature more than the melting temperature.

The scans shown in Fig. 3 were all run at 10°/min. However, similar results were observed at heating and cooling rates of 0.31°/min, i.e., the protein always produced a much larger decrease in transition temperature on cooling than on heating. If this decrease in transition temperature is due to penetration of the protein, it must be partially reversed instantly upon cooling below the transition temperature, while the effect is reversed to a fuller extent only by incubating the vesicles below T_c for a long time. However, the protein exerts some effect even when added to the vesicles below T_c, and this cannot be reversed.

A similar decrease in phase transition was found for basic protein incorporated into phosphatidic acid. Incorporation of 30% basic protein resulted in two peaks appearing in the differential scanning calorimetry (DSC) scan, one at the temperature of pure PA and one decreased by 8°. Incorporation of 50% basic protein (Fig. 4*a* and *b*) resulted in one transition at about 13° lower than pure PA. A greater effect was produced in PA at a lower concentration than for DPPG; however, the effect was not increased for the cooling scan. A similar decrease in T_c of 5° from both the heating and cooling scans was produced in DMPA as shown in Table 2 and Fig. 4*c* and *d*. Thus, brief freezing apparently does not partially reverse the protein effect on PA as it does with DPPG. Even prolonged freezing (12 hr) was not found to decrease the basic protein induced effect on PA. Basic protein binds to PS vesicles causing aggregation and precipitation. However, 30% basic protein had no effect on the melting temperature of PS, although it decreased the freezing temperature giving rise to two peaks at 1.3° and 4.4° lower than pure PS, as shown in Fig. 4*e* and *f* and Table 2. Higher concentrations of basic protein (50%) decreased the freezing temperature by 12° although the peak was obscured by the freezing peak of water. The melting peak could not be measured because of the melting transition of ice. The protein also produces aggregation of PE vesicles but had little effect on T_c even upon cooling. It produced a decrease of 1.4° in PE on heating and cooling at 10°/min, but on cooling at 5°/min it resulted in 2 peaks at 2.6° and 1.5° lower than pure PE as shown in Fig. 4*g* and *h*. These results indicate it penetrates PS to a significant extent only at high concentrations. It probably does not penetrate into PE very greatly. The protein does not

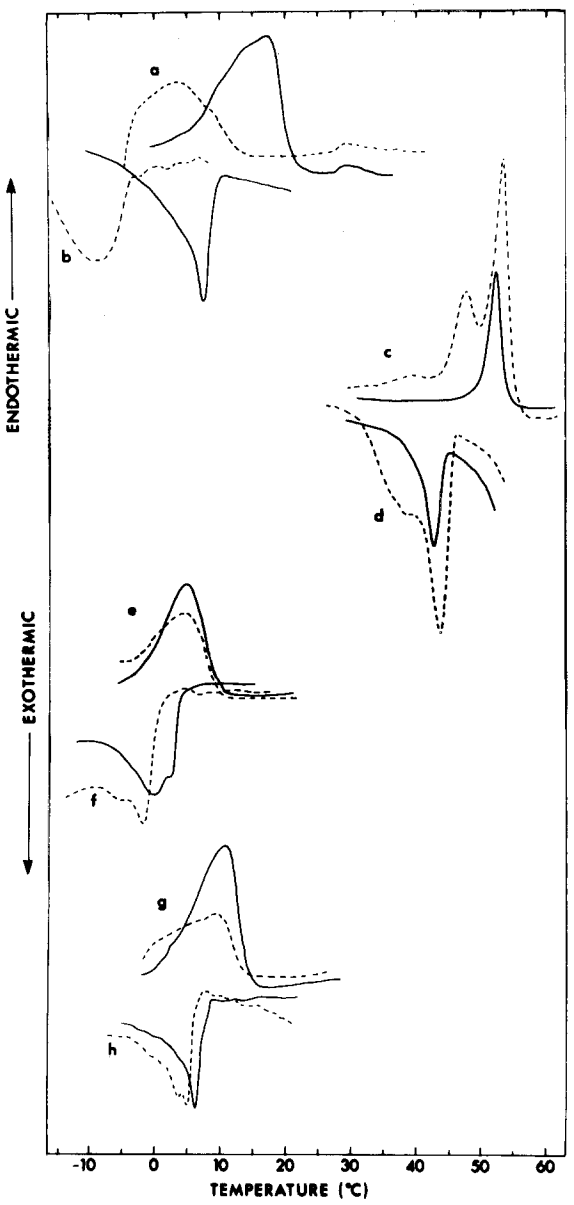


Fig. 4. Differential scanning calorimetry thermograms. (*a*) Heating scans and (*b*) cooling scans for PA (——) and PA + 50 % basic protein (------) at 10°/min; (*c*) heating scans and (*d*) cooling scans for DMPA (——) and DMPA + 20 % basic protein (------) at 10°/min; (*e*) heating scans for PS (——) and PS + 30 % basic protein (------) at 10°/min; (*f*) cooling scans for PS (——) and PS + 30 % basic protein (------) at 5°/min; (*g*) heating scans for PE (——) and PE + 25 % basic protein (------) at 10°/min; (*h*) cooling scans for PE (——) and PE + 25 % basic protein (------) at 5°/min. T_c for heating and cooling are closer if cooling is at 5°/min than at 10°/min. All samples for which DSC scans are shown were prepared by dispersion except for (*g*) and (*h*), which were prepared by dialysis

bind significantly to PC, does not cause precipitation of PC vesicles, and has no effect on the melting or freezing transition temperatures or the premelt temperatures of DMPC or DPPC.

Effect on Fluidity Gradient

In order to understand how basic protein alters the lipid organization resulting in a decrease in T_c and a decrease in the enthalpy (Papahadjopoulos *et al.*, 1975*a*) the lipid vesicles were probed at various depths of the bilayer with a series of fatty acid spin labels with the nitroxide group located at various positions along the fatty acid chain.

The effect of increasing concentration of basic protein on the order parameter S of 5-doxyl-stearate and 12-doxyl-stearate and on the motion parameter τ_0 of 16-doxyl-stearate in DPPG at 50° and PA at 37° are shown in Fig. 5*A* and *B*, respectively. The results indicate that for both lipids basic

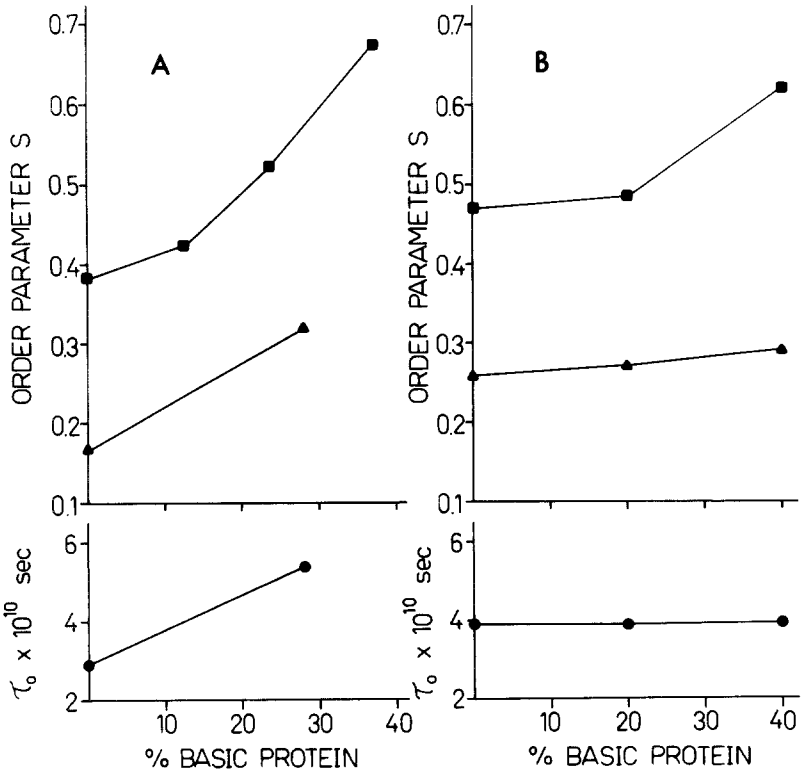


Fig. 5. Dependence of the order parameter S of 5-doxyl-stearate (■), 12-doxyl-stearate (▲), and of motional parameter τ_0 of 16-doxyl-stearate (●) on concentration of basic protein incorporated in vesicles of (*A*) DPPG measured at 50° and (*B*) PA measured at 37°

protein has a much greater effect on fatty acid chain packing near the polar head group region than in the interior of the bilayer. This is particularly true for PA where basic protein has only a small effect on the order parameter of 12-doxyl-stearate and no effect on τ_0 for 16-doxyl-stearate. Indeed, from the spectrum of 16-doxyl-stearate in PA-basic protein (40%) vesicles (Fig. 1 *C* and *D*) it is clear that the protein decreases the order in the interior of the bilayer.

Although it is not valid to calculate order parameters for such rapid motion as observed for 16-doxyl-stearate, it is legitimate to compare such values for similar systems; this is done in Fig. 6 *A* and *B* in order to demonstrate the effect of basic protein on the fluidity gradient throughout the bilayer. It can be seen that for both lipids the fluidity gradient is much steeper in the presence of basic protein. In the case of PA, basic protein increases the fluidity in the interior of the bilayer while decreasing it near the polar head group region. Basic protein decreases the fluidity at all locations in the bilayer for DPPG above the phase transition (50°) but has a much greater effect near the polar head group region of this lipid, also.

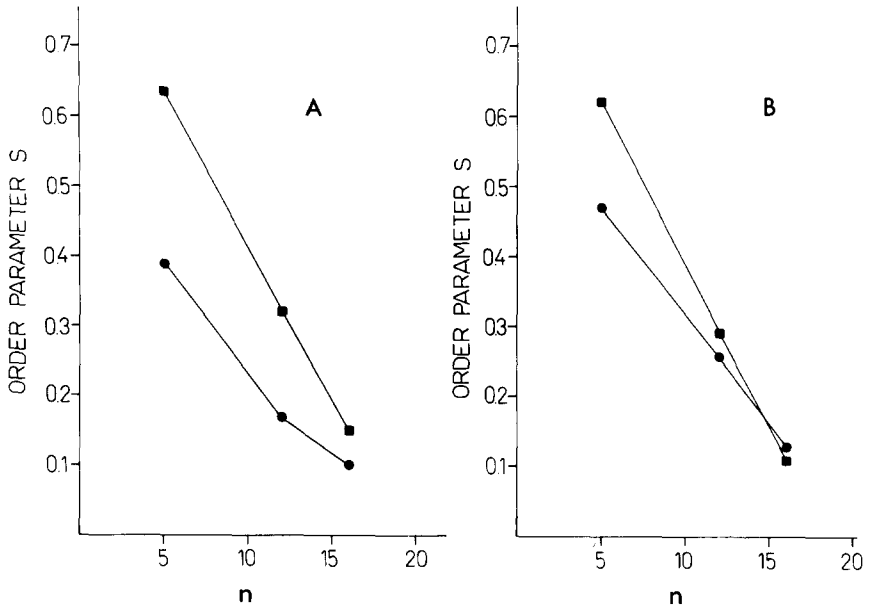


Fig. 6. Dependence of the order parameter *S* on the position of the nitroxide group on the *n*th carbon atom of the fatty acid chain, where *n* is the number of carbon atoms away from the carboxyl group, for (*A*) vesicles of DPPG with 40% basic protein (■) and without basic protein (●) at 50°, and (*B*) vesicles of PA with 40% basic protein (■) and without basic protein (●) at 37°

Effect on Fluidity Above and Below T_c

In order to correlate the effects of basic protein on bilayer fluidity with its effects on T_c when interacting above and below the phase transition, the vesicles prepared by interacting basic protein with preformed sonicated liposomes at 4 °C (Fig. 3) were labeled with the series of fatty acid spin labels. The vesicles were placed in the spectrometer at 28° and the temperature dependence of the spectrum was followed up to 51°. The ESR spectra were then measured again upon cooling back down to 28°. The results are shown in Fig. 7*A*, *B* and *C* for 5-doxyl-stearate, 12-doxyl-stearate and 16-doxyl-stearate, respectively. There is a marked hysteresis in the first heating and cooling curves for vesicles containing basic protein. The cooling curve for the pure lipid is not shown but would be identical to the heating curve, as the heating and cooling rates are much slower than used for DSC. Thus, below T_c on the first heating curve, where the protein is presumably only penetrating the bilayer to a small degree, the protein increases the order parameter throughout the bilayer with its greatest effect being felt in the interior of the bilayer. During the first heating, it decreases T_c by 1.5° for 5-doxyl-stearate to 4° for 16-doxyl-stearate in agreement with the DSC results (Fig. 3). Above T_c, basic protein prevents the large increase in fluidity, as the lipid melts, near the polar head group region but not in the interior of the bilayer, where it has only a small effect on the order parameter of 16-doxyl-stearate. As the phase transition is measured again upon cooling, it can be seen that the presence of the protein prevents recrystallization of the lipid chains at temperatures greater than 28°. Although exact transition temperatures cannot be determined from the graph, it is clear that, on cooling, basic protein decreases the transition temperature much more than on the first heating, in agreement with the DSC results. Furthermore, Fig. 7 shows that after penetration basic protein still increases the order for 5-doxyl-stearate and 12-doxyl-stearate at 28°, although not as much as before melting occurred, but it decreases the order greatly for 16-doxyl-stearate.

Thus, the ESR data indicate that below T_c when basic protein has not yet penetrated into the hydrophobic region to a significant extent, but is bound to the polar head group region by electrostatic interactions, it increases the order throughout the bilayer. After the first melting has occurred, the protein penetrates into the hydrocarbon region of the liquid crystalline phase to a greater extent and has less effect on the order below T_c when the lipid is in the gel state. Below T_c the protein still increases the order near the polar head group region, but decreases it in the interior of the bilayer. Above T_c, when the lipid is in the liquid crystalline phase, basic protein

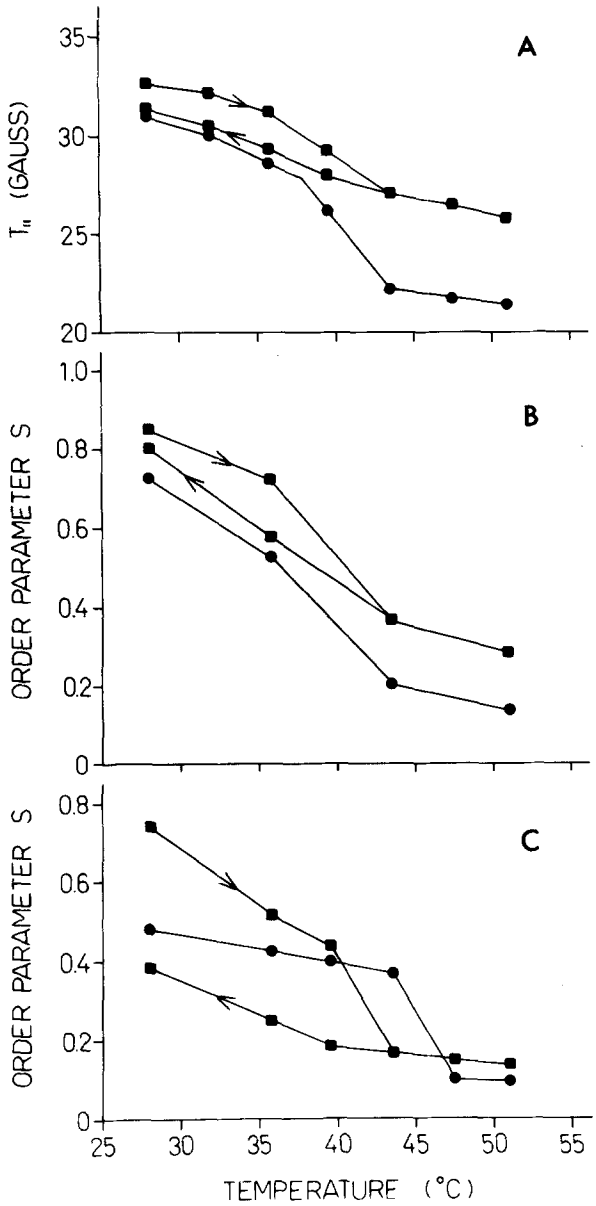


Fig. 7. Dependence on temperature of (*A*) T_{11} for 5-doxyl-stearate and order parameter *S* for (*B*) 12-doxyl-stearate and (*C*) 16-doxyl-stearate in vesicles of DPPG (●) and DPPG + 43% basic protein (■), prepared by adding basic protein in solution to preformed sonicated vesicles at 4 °C. Arrow indicates first heating and cooling scans

increases the order near the polar head group region more than it did in the gel phase and to a much greater extent than in the interior. Indeed, for PA, basic protein decreases the order in the interior even above T_c.

Discussion

Myelin basic protein binds only to acidic lipids, indicating that it initially interacts electrostatically. However, this extrinsic membrane protein can also interact hydrophobically with the lipid fatty acid chains. The ESR results show that the protein greatly reduces the mobility of the fatty acid chains near the polar head group region and thus provide greater evidence for the earlier suggestion based on DSC (Papahadjopoulos *et al.*, 1975*a*) and tryptic hydrolysis (London & Vossenburg, 1973) that portions of the protein penetrate into the bilayer when it is in its liquid crystalline phase. However, unlike another major myelin protein, lipophilin, which is an intrinsic hydrophobic protein, it does not immobilize any lipid as boundary lipid. Freeze fracture of lipid-basic protein vesicles does not reveal the presence of any intramembranous particles (W.J. Vail, *unpublished results*). This is consistent with penetration only partway into the bilayer.

The DSC results indicate that the protein does not penetrate to its full extent when allowed to bind to the lipid in the gel phase. The ESR results show that it increases the order throughout the bilayer in the gel state when it has not yet penetrated, possibly by neutralizing repulsive charges at the polar head group region. When the protein has been allowed to interact with the liquid crystalline phase, it penetrates only partway into the bilayer since it decreases the motion of the fatty acid probe predominantly near the polar head group region. It leaves the fatty acid chains in the interior largely unaffected above the T_c, indicating that the packing density near the polar head group region must be greatly increased in order to accomodate portions of the protein without greatly altering the packing in the interior. Indeed, the protein-induced broadening of the spectrum of 5-doxyl-stearate shows that it disturbs the orientation of the lipid chains near the polar head group region and thus distorts this part of the bilayer. When the vesicles are again cooled down, the presence of portions of the protein within the bilayer increases the fluidity in the interior of the bilayer because the lipid chains cannot crystallize around the protein. The fatty acid chains of DPPG may not be able to crystallize until at least part of the protein is frozen out at temperatures below 30° so that the effect on the melting temperature is not as great as on the freezing temperature.

Thus, basic protein, while having little effect on the lipid bilayer at low concentrations, has major effects on the bilayer structure at higher concentrations. It decreases the phase transition temperature and the enthalpy by penetrating partway into the bilayer, distorting the packing and

decreasing the freedom of motion of the lipid chains near the polar head group region while having little effect on the chain motion in the interior of the bilayer. Penetration of the protein prevents crystallization of the lipid chains until a lower temperature is reached, at which point some portions of the protein may be instantly frozen out while other portions are not frozen out until after prolonged freezing. This penetration causes monolayer expansion and increases the permeability of the bilayer.

Another protein, cytochrome *c*, which was found to have similar effects in decreasing T_c and the enthalpy, ΔH, increasing permeability, and causing monolayer expansion (Papahadjopoulos *et al.*, 1975*a*), was found to have little or no effect on the ESR spectra of the series of fatty acid spin labels used here (Van & Griffith, 1975). However, the two studies were carried out at different pH and using different lipids which may account for the discrepancy.

Although basic protein has 25% basic amino acids dispersed throughout its sequence, it also has short sections of apolar amino acid dispersed throughout, and these may be the portions of the chain which penetrate into the hydrocarbon region. London and Vossenburg (1973) found that the protein was protected from trypsin hydrolysis at some, but not all, normally susceptible sites in the region amino acids 20–113 while the rest of the protein was not protected from hydrolysis, thus indicating that it is portions in this part of the protein which are penetrating into the lipid bilayer. The N-terminal peptide 1–116 increased the surface pressure of monolayers more than the C-terminal 117–170, but this portion did have some effect also, indicating that part of it may also penetrate. Human myelin basic protein has 10 segments of at least 5 neutral and/or hydrophobic amino acids uninterrupted by polar amino acids. These include amino acids 14–21, 26–30, 60–64, 66–73, 85–90, 98–102, 108–112, 114–118, 123–129 and 163–168. There are 5 peptides of at least 4 amino acids without even a hydroxyl group, 26–30, 44–47, 61–64, 85–90 and 123–126. These segments would be sufficiently long to form a loop penetrating into the hydrocarbon region.

Calculation of the probability of β-bends in basic protein indicated that there was a high probability (greater than 10^{-4}) at many sites throughout the protein, with an especially high probability (greater than 4×10^{-4}) at 6 sites in the region 0–100 and at one site in the remainder near the carboxyl terminal (Epand *et al.*, 1974).

The varying behavior observed with different lipids indicates that, although the protein can bind electrostatically to all negatively charged lipids, its additional interaction and penetration depends on the lipid. It apparently can penetrate into PA and DPPG to a significant extent, PS to a

lesser extent, and PE very little. The small effect on PS is consistent with the earlier observation by London and Vossenburg (1973) that PS does not protect basic protein against tryptic hydrolysis while other lipids do. It also has less effect on monolayers of PS and PE than of other lipids (Demel *et al.*, 1973). However, it does increase the permeability of liposomes of PS to Na^+ (Papahadjopoulos *et al.*, 1975*a*), indicating some penetration into this lipid. The decrease in T_c upon cooling and the broadening effect on the ESR spectrum are consistent with this. However, it must be frozen out after cooling since no decrease is seen upon reheating. The lack of broadening effect on the ESR spectrum of PE also indicates that it does not penetrate this lipid, but increases the order by binding to the polar head group region as other proteins which bind electrostatically have been observed to do (Yu, Baldassare & Ho, 1974).

The fact that basic protein binds at all to PE and not to PC is yet another example of the different behavior of these two lipids, which have similar polar head groups and a net neutral charge at pH 7.4. However, the ethanolamine moiety can interact intermolecularly by hydrogen bonding with adjacent PE molecules, while PC, which is a zwitterion, can only interact ionically by charge neutralization. This may allow the ethanolamine moiety to be oriented parallel to the bilayer surface (Phillips, Finer & Hauser, 1972), exposing some negative charges for binding to positively charged groups on the protein or divalent cations. The intermolecular interactions at the polar surface of PE vesicles may predominate, however, and prevent penetration of the protein. The choline of PC, however, may be oriented perpendicular to the bilayer (Levine *et al.*, 1972), thus shielding the negatively charged phosphate and preventing binding of basic proteins or divalent cations. Several studies indicate that the molecular motion of the PE head group is more restricted than the PC head group (Michaelson, Horowitz & Klein, 1974; Seelig & Galley, 1976). NMR studies suggest that the dihedral angles of the head group are more variable in PC than in PE (Akutsu & Kyogoku, 1977; Gally, Niederberger & Seelig, 1975), indicating greater flexibility of the choline moiety. Although positively charged proteins or ions do not bind to PC in the absence of a negatively charged constituent, negatively charged proteins such as poly(L-glutamic acid) and poly(L-tyrosine) do bind to PC (Yu *et al.*, 1974).

The ESR and DSC results indicate that basic protein may be able to penetrate deeper into the bilayer of DPPG than PA. The protein decreases the motion of 16-doxyl-stearate in PG, but increases it in PA. Furthermore, part of the effect in decreasing T_c in DPPG is reversed even upon brief cooling but not in PA or DMPA. Thus, the additional portions which

penetrate in PG are frozen out upon brief cooling but can repenetrate when the vesicles are again taken above T_c. If various portions of the protein can penetrate DPPG depending on the fluidity of the bilayer, this may account for the presence of two peaks in the DSC heating scan at high concentrations, both of which occur at lower temperatures than for pure DPPG. In the case of PA, two peaks are also seen, but one is at the same temperature as pure PA and thus probably represents lipid which is not interacting with the protein.

This variable interaction and penetration of basic protein into different lipids means that the sites which are exposed to the aqueous phase would depend on the lipid composition of the membrane. Thus, the lipid composition of the membrane could determine whether antigenic sites are exposed and thus alter the susceptibility of myelin to attack by antibodies or sensitized lymphocytes. It might also account for the fact that the antigenic sites of the protein vary greatly from one species to another (Eylar, 1972).

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