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Interaction of myelin basic protein and polylysine with synthetic species of cerebroside sulfate

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The effect of myelin basic protein on the myelin lipid cerebroside sulfate was studied by differential scanning calorimetry and use of the fatty acid spin label, 16-S-SL, in order to determine (i) the effect of basic protein on the metastable phase behavior experienced by this lipid, and (ii) to determine if basic protein perturbs the lipid packing as it does with some acidic phospholipids. The effects of basic protein on the thermodynamic parameters of the lipid phase transition were compared with those of polylysine which has an ordering effect on acidic phospholipids as a result of its electrostatic interactions with the lipid head groups. Different synthetic species of cerebroside sulfate of varying fatty acid chain length and with and without a hydroxy fatty acid were used. The non-hydroxy fatty acid forms of cerebroside sulfate undergo a transition from a metastable to a more ordered stable state while the hydroxy fatty acid forms remain in the metastable state at the cation concentration used in this study (0.01 M Na^+ or K^+). The non-hydroxy fatty acid forms were still able to go into a stable state in the presence of both basic protein and polylysine. At low concentrations, basic protein increased the rate of the transition to the stable state, while polylysine decreased it for the longest chain length form studied. However, at high concentrations, basic protein probably prevented formation of the stable state. The hydroxy fatty acid forms did not go into the stable state in the presence of basic protein and polylysine. It is argued that the increased rate of formation of the stable state in the presence of basic protein and decreased rate in the presence of polylysine are consistent with interdigitation of the lipid acyl chains in the stable state. Basic protein also had a small perturbing effect on the lipid. It decreased the total enthalpy of the lipid phase transition. When added to the non-hydroxy fatty acid forms it increased the temperature of the liquid crystalline to metastable phase transition and decreased the temperature of the stable to liquid crystalline phase transition. It significantly decreased the transition temperature of the hydroxy fatty acid forms but only a portion of the lipid was affected. In contrast, polylysine increased the transition temperature of the metastable and stable states of all forms of cerebroside sulfate but had a greater effect on the non-hydroxy fatty acid forms than on the hydroxy fatty acid forms. These results suggested that basic protein does not have as great a perturbing effect on cerebroside sulfate as it does on some acidic phospholipids. This reduced perturbing effect is attributed to the participation of cerebroside sulfate in intermolecular hydrogen bonding interactions.

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Abbreviations: NFA, non-hydroxy fatty acid; HFA, hydroxy fatty acid; CBS, cerebroside sulfate; P-CBS, S-CBS, L-CBS, palmitoyl, stearoyl, and lignoceroyl forms, respectively, of cerebroside sulfate; BP, myelin basic protein; DSC, differential scanning calorimetry; ESR, electron spin resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Introduction

Myelin basic protein is an extrinsic protein which has a perturbing effect on the bilayer of some lipids causing, among other effects, a decrease in the temperature and enthalpy of the phase transition [1–5]. The degree of perturbation caused by the protein depends on the lipid polar head-group [3,6,7]. Participation of the lipid head-groups in intermolecular hydrogen bonding has been concluded to prevent the expansion and lateral separation of the lipid molecules caused by basic protein, possibly by penetration of some hydrophobic residues part way into the bilayer or by a more complex mechanism [3,5,7].

Cerebroside sulfate is an acidic lipid which should be able to hydrogen bond intermolecularly like cerebroside, via the hydroxyl groups on the galactose, hydroxyl groups present on the fatty acid chain and the sphingosine base, and the amide moiety [8]. Previous studies of the effect of basic protein on cerebroside sulfate showed that the protein had a less perturbing effect on this lipid than on other acidic lipids [3,7] and that antigenic determinants of the protein were more exposed to antibody than when the protein was bound to other acidic lipids [9], consistent with its intermolecular hydrogen bonding properties.

These earlier studies were done using natural bovine brain cerebroside sulfate, which is heterogeneous in fatty acid chain length and contains a mixture of hydroxy and non-hydroxy fatty acids. We recently prepared synthetic forms of cerebroside sulfate containing either hydroxy or non-hydroxy fatty acids of specified length in order to study the contribution of the fatty acid hydroxyl group to intermolecular hydrogen bonding interactions and its effect on the lipid phase behavior [10]. Study of these synthetic forms by differential scanning calorimetry and use of spin labels revealed much more information about their phase behavior than was obtained by study of the natural form [11].

These lipids undergo metastable phase behavior, which is probably a consequence of the unequal chain length between the sphingosine base and the acyl chain. The transition from a metastable state to a more ordered stable state is inhibited by the fatty acid hydroxyl group and by Li^+ and divalent cations; this inhibitory effect is counteracted by an increase in fatty acid chain length. The structure of the stable state has not been determined, although its behavior suggests that it may be interdigitated; however, changes in hydration are also a possible mechanism for the metastability [11]. The fatty acid hydroxyl group causes an increase in the temperatures of the transitions to the liquid crystalline phase of both the metastable and the stable states, indicating that it strengthens the intermolecular hydrogen bonding interactions. In the present study, the effect of myelin basic protein and a polyvalent cation, polylysine, on the phase behavior of the hydroxy and non-hydroxy fatty acid forms of cerebroside sulfate was studied by DSC and by the use of a fatty acid spin label.

Methods and Materials

Cerebroside sulfate containing palmitic, stearic, or lignoceric acids (non-hydroxy fatty acid) or α-hydroxypalmitic and α-hydroxystearic acids (hydroxy fatty acid) were prepared as described earlier [10,12]. Basic protein was extracted from isolated human myelin as described [13] and stored in the lyophilized form. The fatty acid spin label 16-S-SL was purchased from Syva (Palo Alto, CA). Polylysine was purchased from Sigma.

The lipid was dispersed at 70°C by vortex mixing in buffer at a concentration of 1.2 mg lipid/50 μl Hepes buffer (10 mM) containing 10 mM NaCl and 1 mM EDTA at pH 7.4. For samples with protein, an aliquot of the protein solution at a concentration of 0.5 mg/100 μl in the same buffer was then added and the sample was vortexed again at a temperature of 65°C. The pH of the protein solution was adjusted back to 7.4 if necessary before adding to the lipid. The sample was centrifuged in an Eppendorf bench centrifuge, most of the supernatant was removed and the pH was checked, and the pellet was loaded into an aluminum DSC pan. The sample was prepared similarly for ESR measurements except that the lipid and spin label were first dissolved together in chloroform/methanol (2 : 1, v/v) at a 200 : 1 mole ratio and the solvent was evaporated under nitrogen. The sample was loaded into a 50 μl capillary tube which was sealed and centrifuged

at 2000 rpm for 10 min. The lipid-basic protein ratio in the pellets was determined by taking aliquots for lipid determination by galactose analysis [14] and protein determination by amino acid analysis on a Durrum D-500 amino acid analyzer after hydrolysis with 5.7 M HCl for 19 h at 100°C. Polylysine was added at a 1 : 3 weight ratio to the lipid.

Calorimetric measurements were made on a Perkin-Elmer DSC-2 differential scanning calorimeter equipped with a Perkin-Elmer data station. Heating and cooling rates of 1.25–10 deg. C/min were used on all samples. All scans were reversible and reproducible through many heating and cooling cycles unless otherwise noted. The temperature of maximum heat capacity was defined as the phase transition temperature, T_m. The transition temperatures obtained on cooling were corrected for instrumental hysteresis. For heating and cooling rates of 10 deg. C/min, the correction factor was found to be 4.5 deg. C by using lipids which do not undergo metastable phase behavior, such as synthetic, symmetric phospholipids or cerebroside sulfate in the presence of Li^+ and divalent cations [11]. Any difference in T_m between heating and cooling scans which remains after this correction, as for the cerebroside sulfate samples which undergo metastable phase behavior, indicates the true difference in T_m between the transitions observed on heating and cooling. The peak areas were determined by use of the data station and the amount of lipid in the pan was determined by galactose analysis so that the enthalpy of the phase transition could be calculated [11]. The baseline under the peaks was extrapolated by a straight line connecting the baseline on either side of the peaks; the extrapolation was carried out by the data station after choosing the points to be connected.

ESR spectra were measured on a Varian E-104B ESR spectrometer equipped with a Varian temperature control accessory. The microwave power used was 10 mW. The motional parameter, τ_o, and maximum hyperfine splitting, T_{max}, were measured as described earlier [3].

Results

The effect of basic protein on the phase transition of non-hydroxy and hydroxy fatty acid forms of cerebroside sulfate containing fatty acids of varying chain length was determined in order to monitor the perturbing effect of basic protein on the lipid bilayer and its effect on the metastable phase behavior of this lipid. In the case of acidic phospholipids, the perturbing effect of basic protein on the lipid chain packing results in a decrease in the temperature and enthalpy of the lipid phase transition [1,3]. If only part of the lipid is affected, two peaks may be observed, one at a lower temperature and one at a temperature similar to that of the pure lipid [4]. For some lipids the protein has a greater effect on the freezing temperature than the melting temperature [3,4]. In contrast to basic protein, polylysine increases the phase transition temperature of acidic phospholipids, in-

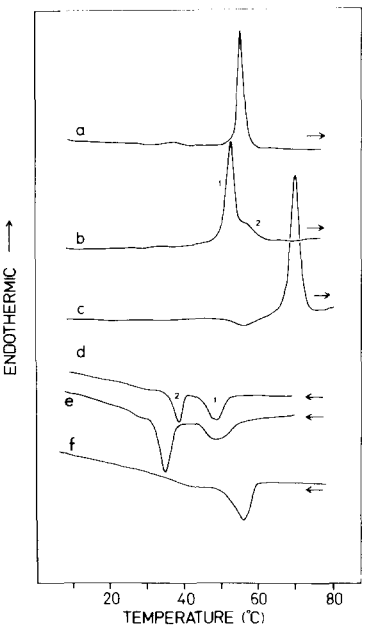

Fig. 1. DSC scans of NFA-L-CBS in 0.01 M Na^+ at heating and cooling rates of 10 deg. C/min. a–c, heating scans; d–f, cooling scans. Arrows indicate the direction of change in temperature. (a,d) lipid only; (b,e) 21 wt.% basic protein; (c,f) 30 wt.% polylysine. Sensitivity settings in mcal/s were (a,d) 1.5; (b,e) 0.8; (c,f) 1.0. Different amounts of lipid were present for each sample so the peak areas cannot be directly compared except for heating and cooling scans for the same sample. Enthalpies are given in Table I. Instrumental hysteresis causes the T_m to occur at a 4.5 deg. C lower temperature on cooling than on heating at a rate of 10 deg. C/min. The temperatures in Table I have been corrected for this hysteresis but the scans in the figures have not.

dicating that it has an ordering effect on the lipid [1,4].

However, the situation is more complicated in the case of cerebroside sulfate, whose metastable phase behavior also results in multiple transitions which can be detected by DSC as shown in Fig. 1a and d for NFA-L-CBS in 0.01 M Na^+. On heating (Fig. 1a), only a single endothermic transition is observed at 54.8°C with an enthalpy of 14.8 kcal/mol (Table I). This is the transition of the stable state to the liquid-crystalline phase [11]. On cooling, the liquid-crystalline phase freezes first into a metastable state at a somewhat lower temperature, 53.2°C (peak 1 in Fig. 1d) and with a lower enthalpy, 7.8 kcal/mol. As the sample is cooled further the metastable state goes into the stable state (peak 2 in Fig. 1d) with further release of heat, 5.9 kcal/mol (Table I). This behavior is reversible and reproducible.

Basic protein altered the phase transitions of all forms of cerebroside sulfate to some extent, both on heating and cooling, sometimes giving new peaks at lower temperatures, while polylysine increased the transition temperature of all forms of cerebroside sulfate. (DSC thermograms are shown in Figs. 1, 2, 4–6, 8, 9, and each will be discussed in more detail below.) However, it was necessary to determine whether the multiple transitions of the BP-CBS complex were a result of perturbation of a portion of the lipid, as for acidic phospholipids, or participation of the lipid-protein complex in the same kind of metastable phase behavior

TABLE I

EFFECT OF BASIC PROTEIN (BP) AND POLYLYSINE ON T_m AND ΔH OF PHASE TRANSITIONS OF CEREBROSIDE SULFATE

All samples in the presence of 0.01 M Na^+ except with K^+ (0.01 M). Heating and cooling rates were 10 deg. C/min.

Sample	Heat		Cool [a]	
	T_m (°C)	ΔH (kcal/mol) [b]	T_m (°C)	ΔH (kcal/mol) [b]
NFA-P-CBS	50.2	8.5 ± 0.5	50.3	8.1 ± 0.1
+ BP (44%)	40, <u>50.7</u> [c]	3.1, 4.9 (8.0)	36.5, <u>48.8</u> [c]	1.5, 5.6 (7.0)
+ polylysine	66	7.4	57.9	7.6
HFA-P-CBS	53.2	6.4 ± 0.7	52.8	6.1 ± 0.7
+ BP (33%)	41, <u>51</u> [c]	2.1, 3.5 (5.6)	36.5, <u>49.5</u> [c]	1.4, 3.8 (5.2)
NFA-S-CBS 0.01 M Na^+	52	13.8 ± 0.9	42.3, <u>50.5</u> [c]	0.3, 7.4 (8.1 ± 0.7)
+ BP (10%)	52	13.9	43.8, 52.3	4.8, 8.5 (13.3)
+ BP (19%)	<u>49</u> [c], 53	7, 2.6 (9.5)	<u>41.2</u> [c], 51.5	5.2, 4.9 (10.1)
+ BP (33%)	<u>48.6</u> [c], 52	5, 2.1 (7.1)	36.1, 48.7	4.2, 4.4 (8.6)
+ polylysine	67	7.8	57.9	8.6
NFA-S-CBS 0.01 M K^+	55.1	13.8 ± 2.0	51.3	8.3 ± 1.2
+ BP (12.4%)	54.4	11.3	40, 52.6	0.9, 7.8 (8.7)
+ BP (21.7%)	55.9	9.6	42.3, 53.9	5.2, 4.6 (9.8)
HFA-S-CBS	56.4	6.4 ± 0.5	56.4	6.4 ± 0.8
+ BP (19%)	40.6, 56.9	1.3, 2.9 (4.2)	39.5, <u>55.6</u> [c]	1.2, 3.3 (4.5)
polylysine [d] (1)	61.3, 66.6	2.4, 3.3 (5.7)		
(2)	60.4	5.1	58.5	5.4
NFA-L-CBS	54.8	14.8 ± 1.6	43.2, 53.2	5.9, 7.8 (14.9)
+ BP (21%)	<u>52.4</u> [c], 56.8	9.1, 2.4 (11.9)	39.6, 54	5.7, 3.9 (12.0)
+ polylysine	70.7	18.4	61.2	13.3

[a] T_m on cooling has been corrected for instrumental hysteresis.

[b] Value in parentheses is total enthalpy. Other values are of peak 1 and peak 2.

[c] Temperature of largest peak is underlined.

[d] First and repeated heating scans.

experienced by the pure lipid. Use of a fatty acid spin label helped to distinguish between these two mechanisms.

The amount of basic protein actually bound to each lipid was analysed and was found to decrease with increase in chain length as shown in Table II. Furthermore, less protein bound to the hydroxy fatty acid forms than to the non-hydroxy fatty acid forms and the difference was greater for the longer chain length form HFA-S-CBS than HFA-P-CBS. The mechanism for the effect of basic protein on the phase transition could be determined most clearly for NFA-L-CBS and NFA-S-CBS and the study of these lipids helped to understand the results obtained with the shorter chain length and hydroxy fatty acid forms of cerebroside sulfate. Therefore, the effect of basic protein and polylysine on NFA-L-CBS and NFA-S-CBS will be described first.

The effect of basic protein on the phase transition of NFA-L-CBS in 0.01 M Na^+ is shown in Fig. 1b and e. The main transition (peak 1) of the basic protein complex occurred at a somewhat lower temperature than the pure lipid and a broad peak (peak 2) was also present at a slightly higher temperature (Fig. 1b and Table I). The total enthalpy was decreased. The increase in T_m of the broad component (peak 2) relative to the pure lipid can be seen more easily at a slower heating rate, 5 deg. C/min, as shown in Fig. 2a,b. On cooling (Fig. 1e), the scan resembled that of the pure lipid except that the first transition (peak 1) was broader and of lower enthalpy than for the pure lipid, and the second (peak 2) occurred at a lower temperature but with a similar enthalpy (Table I). This suggested that the same kind of metastable phase behavior experienced by the pure lipid occurred in the presence of basic protein and that the sharp main transition in Fig. 1b is the stable state to liquid-crystalline phase transition. The lower temperature of the main transition of the BP-CBS complex relative to pure cerebroside sulfate indicates that it is a transition of protein-bound lipid and not of residual unbound lipid.

The designation of peak 1 in Figs. 1b and 2b as the transition of the stable state of the BP-CBS complex is further supported by the DSC scans obtained when heating from a temperature above 30°C rather than −3°C as for Fig. 1b and 2b. If the sample is only cooled to 37°C before reheating, the height of the first sharp peak decreases (Fig. 2c). If the sample is cooled only to 42 or 47°C before heating, primarily the broad peak at 55°C is observed (Fig. 2d,e), indicating that the sample has not yet gone into the stable state. The pure lipid has the same behavior (not shown).

TABLE II

AMOUNT OF BASIC PROTEIN INCORPORATED INTO CEREBROSIDE SULFATE [a]

If a standard deviation is given, data represent an average of two or three determinations. If no standard deviation is given, only one measurement was made.

Lipid	% BP added	% BP bound
NFA-P-CBS	30	20
	50	44 ± 2
HFA-P-CS	30	16.5
	50	33 ± 5
NFA-S-CBS		
0.01 M Na^+	10	10
	30	19
	50	33 ± 0.3
0.01 M K^+	30	12.4
	50	21.7
HFA-S-CBS	50	19
NFA-L-CBS	50	21 ± 1

[a] % BP of total weight. All in 0.01 M Na^+ except as noted.

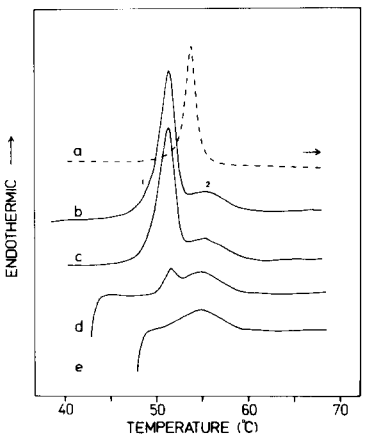

Fig. 2. DSC heating scans of NFA-L-CBS in 0.01 M Na^+ alone (a) and in the presence of 21 wt.% basic protein (b–e) at a heating rate of 5 deg. C/min. Heated from (a,b) −3°C; (c) 37°C; (d) 42°C; (e) 47°C. Sensitivity settings in mcal/s were (a) 0.8; (b–e) 0.3. The same sample was used for scans b–e.

However, in the presence of basic protein part of the lipid remains in the state which undergoes the broad higher temperature transition even after conversion of most of the sample to the stable state (as in Fig. 1b). This transition also must be a transition of protein-bound lipid, since it occurs at a higher temperature than the metastable to liquid crystalline phase transition of the pure lipid and since the pure lipid goes completely into the stable state under these conditions. It may represent a part of the sample which contains more bound basic protein than the portion which goes into the stable state. Thus, a high concentration of bound basic protein may prevent the lipid from going into the stable state.

Polylysine increased the temperature and enthalpy of the main endothermic transition of NFA-L-CBS (Table I and Fig. 1c). An exothermic transition occurred before the endothermic transition in the heating scan. The T_m of the endothermic transition was similar to that observed earlier in the presence of high concentrations of K^+ [11]. On cooling (Fig. 1f), the temperature of the main transition was 10 deg. C below that observed on heating, after correction for instrumental hysteresis, and the enthalpy also was reduced. (The temperatures in Table I have been corrected for instrumental hysteresis, while the scans in the figures have not). A second broad transition at 40–46°C was also observed on cooling. The total enthalpy of both transitions observed on cooling at 10 deg. C/min was considerably less than that observed on heating. The lower temperature and enthalpy of the freezing transition indicated that the sample went into the metastable phase on freezing, rather than into the stable phase, when cooled rapidly (10 deg. C/min) as for Fig. 1f. The transition to the stable phase occurred during the exothermic transition observed on reheating as in Fig. 1c. Thus, the NFA-L-CBS complex with polylysine went into the stable phase at a slower rate than the pure lipid or its complex with basic protein.

We found earlier that the fatty acid spin label, 16-S-SL, is squeezed out of the stable phase of cerebroside sulfate in the presence of Na^+ or K^+ into solid domains, giving an exchange broadened component, and into the aqueous phase, giving an isotropic mobile component (see Fig. 4 in Ref. 11). The spin label is soluble in the less ordered metastable phase and gives a spectrum characteristic of anisotropic motion. The spectra of 16-S-SL in the BP-NFA-L-CBS complex at different temperatures is shown in Fig. 3. At 42°C, between the temperatures pf peaks 1 and 2 observed on cooling in Fig. 1e, the spin label has the anisotropic motion characteristic of the metastable phase of the pure lipid (Fig. 3A). On cooling to 38°C, below the temperature of peak 2, a large change in the spectrum occurs as shown in Fig. 3b. The spectrum indicates a decrease in the anisotropy of the motion and a lower order parameter. The spectrum is also decreased in height indicating that a decrease in rotational motion also occurs. On cooling further to 30°C, a component with greater hyperfine splitting appears as indicated by arrows in Fig. 3C, and at 9°C the spectrum is a powder spectrum with T_{max} = 30.3 G, characteristic of relatively slow motion (Fig. 3D).

In the pure lipid a much greater reduction in height occurs on lowering the temperature below that of peak 2, as a result of the exchange broad-

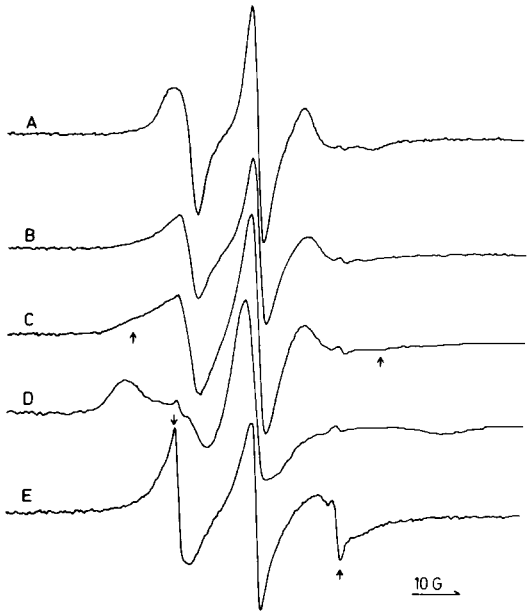

Fig. 3. ESR spectra of 16-S-SL in NFA-L = CBS in 0.01 M Na^+ in the presence of 21% basic protein at different temperatures (A–D): A, 42°C (metastable); B, 38°C (stable); C, 30°C (stable); D, 9°C (stable). A and B are plotted out to the same scale for comparison of the spectral height, while C and D are scaled up. E, pure NFA-L-CBS in the presence of 0.01 M Na^+ at 9°C (stable).

ening [11]. The spectrum of 16-S-SL in the stable state of pure NFA-L-CBS at 9°C is shown in Fig. 3E for comparison with 3D. The sharp peaks resulting from spin label squeezed out into the aqueous phase can be seen (indicated by arrows). Spectral subtraction was performed in our earlier study in order to demonstrate the exchange broadened component [11]. In contrast, the stable state in the presence of basic protein must be disordered enough to allow solubility of the spin label.

NFA-L-CBS does not form the metastable phase at temperatures below 30–40°C; however, the spectrum of 16-S-SL in the metastable phase of HFA-S-CBS at 9°C and 30°C can be seen in Fig. 7A,B for comparison with that in the stable phase of the BP-CBS complex in Fig. 3C,D at those temperatures. (Chain length was found to have little effect on the spectrum provided the lipid was in the same state.) At 30°C, the spin label has anisotropic motion in the metastable state of the pure lipid, while at 9°C, T_{max} is 26.5 G (Fig. 7A,B), considerably less than that in the basic protein-cerebroside sulfate complex (Table III), indicating greater rotational motion in the metastable phase. Thus, the spectrum in the stable phase of the BP-CBS complex can be distinguished from that of the metastable phase of the pure lipid. The behavior of 16-S-SL in the complexes of basic protein with other forms of cerebroside sulfate helped to determine whether the sample was in the stable or metastable state.

TABLE III

EFFECT OF CATIONS, BASIC PROTEIN, AND POLYLYSINE ON ESR SPECTRAL PARAMETERS OF 16-S-SL IN CEREBROSIDE SULFATE

	T_{max} (G) at 9°C	State at 9°C	τ_0 (ns) at 64°C
NFA-P-CBS			
Na^+	[a]	stable	0.71
K^+	[a]	stable	0.95
BP	29.9	stable	1.06
NFA-S-CBS			
Na^+	[a]	stable	0.79
K^+	[a]	stable	1.00
Ca^{+2}	26.0	metastable	1.10
BP	29.4	stable	1.14
NFA-L-CBS			
Na^+	[a]	stable	0.92
BP	30.32	stable	1.36
HFA-S-CS			
Na^+	26.5	metastable	0.87
K^+	26.0	metastable	[b]
BP	28.5	metastable	1.29

[a] Spin label is squeezed out.

[b] Below transition to liquid-crystalline phase.

DSC scans of NFA-S-CBS alone (Fig. 4a,f) and with varying amounts of basic protein and polylysine in 0.01 M Na^+ are shown in Fig. 4. On cooling, pure NFA-S-CBS does not go into its stable phase in as cooperative a transition as NFA-L-CBS. The enthalpy of the metastable to stable state transition (peak 2 on cooling) of pure NFA-S-CBS increases as the cooling rate is decreased and as the Na^+ concentration is increased [11]. By measuring the effect of basic protein on

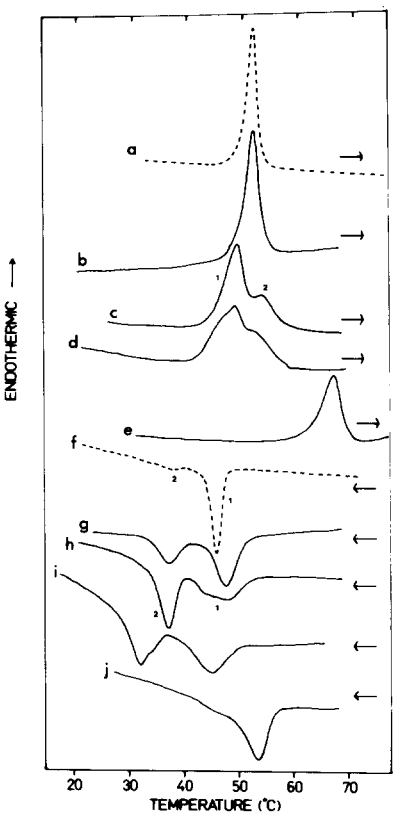

Fig. 4. DSC scans of NFA-S-CBS in 0.01 M Na^+ at heating (a–e) and cooling (f–j) rates of 10 deg. C/min. (a,f) lipid only; (b,g) 10 wt.% basic protein; (c,h) 19 wt.% basic protein; (d,i) 33 wt.% basic protein; (e,j) 30 wt.% polylysine. Sensitivity settings in mcal/s were (a,f) 1.0; (b,g) 0.5; (c,h) 0.5; (d,i) 0.3; (e,j) 0.5. Other details as in caption to Fig. 1.

the enthalpy of peak 2 at different cooling rates, the effect of basic protein on the rate of the transition from the metastable to the stable state can be determined. At a cooling rate of 10 deg. C/min and a Na^+ concentration of 0.01 M (Fig. 4f), the enthalpy of peak 2 for the pure lipid is only 0.3 kcal/mol out of a maximum of 5–6 kcal/mol (Table I), indicating only partial conversion to the stable state during this transition.

As for NFA-L-CBS, basic protein decreases the temperature of the stable to liquid crystalline phase transition (peak 1) of NFA-S-CBS, induces a second broad component at a higher temperature (peak 2) observed in heating scans (Fig. 4b–d and Table I) and decreases the total enthalpy. These effects increase with increasing protein content. On cooling, basic protein increases the temperature and decreases the enthalpy of peak 1, the liquid crystalline to metastable phase transition, especially at low protein concentrations, and significantly increases the enthalpy of peak 2, the metastable to stable state transition to about 5 kcal/mol, close to its maximum value, even at a relatively fast cooling rate of 10 deg. C/min (Fig. 4g–i, Table I). These results indicate that low concentrations of basic protein increase the rate of the transition to the stable state. However, high concentrations inhibit formation of the stable state, since a portion of the lipid remains in the metastable state at 19 and 33% basic protein content (Fig. 4c,d).

The spectra of 16-S-SL in the complex of NFA-S-CBS with basic protein (not shown) were similar to those in the NFA-L-CBS complexes (Fig. 3), thus confirming that NFA-S-CBS also went into the same kind of stable phase with basic protein as NFA-L-CBS. T_{max} of 16-S-SL in the basic protein complex at 9°C was greater than that of the metastable states (Table III).

Polylysine increased the transition temperature of NFA-S-CBS but not the enthalpy (Fig. 4e and Table I). On cooling, the transition occurred at a 10 deg. C lower temperature than on heating, indicating that the complex refroze into the metastable state (Fig. 4j and Table I). A second broad peak occurred at 43–50°C. The total enthalpy of both transitions observed on cooling equalled that on heating so that the second lower temperature transition must be due to the transition to the stable phase. Thus, the complex of polylysine with NFA-S-CBS goes into the stable phase faster than its complex with NFA-L-CBS.

In order to confirm the stimulating effect of basic protein at low concentrations on the rate of the transition from the metastable to the stable state, the effect on NFA-S-CBS in the presence of 0.01 M K^+ was also studied. The rate of this transition is slower in the presence of K^+ than Na^+ [11] and thus no exothermic transition at a temperature below the freezing transition from the liquid crystalline phase (peak 1) is observed at a cooling rate of 10 deg. C/min as shown in Fig. 5b (an exothermic transition is observed at slower rates). Following a cooling scan at 10 deg. C/min, an exothermic transition before the main high enthalpy endothermic transition is observed on heating (Fig. 5a), indicating that conversion to the stable state occurs during the heating scan. Less basic protein was bound than in the presence of

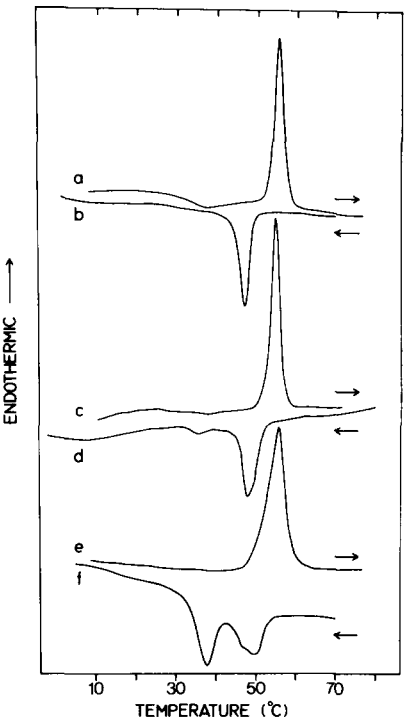

Fig. 5. DSC scans of NFA-S-CBS in the presence of 0.01 M K^+. (a,c,e) heating scans and (b,d,f) cooling scans at 10 deg. C/min. (a,b) no basic protein; (c,d) 12.4% basic protein; (e,f) 21.7% basic protein. Sensitivity settings in mcal/s were (a,b) 0.8; (c–f) 0.5. Other details as in caption to Fig. 1.

Na^+ (Table II), probably because of the higher affinity of K^+ for cerebroside sulfate relative to Na^+. 12.4 and 21.7% basic protein have little effect on the T_m but decrease the enthalpy of the stable to liquid crystalline state transition (Fig. 5c,e, Table I). Basic protein increases the temperature and decreases the enthalpy of the liquid crystalline to metastable state transition seen on cooling (peak 1), causes the appearance of the lower temperature exothermic transition (peak 2) at a cooling rate of 10 deg. C/min (Fig. 5d,f) and abolishes the exothermic transition on reheating (Fig. 5e). The enthalpy of peak 2 on cooling increases with protein concentration (Table I). These results indicate that in the presence of the protein in 0.01 M K^+, the transition to the stable state occurs on cooling at a rate of 10 deg. C/min, while it did not for the pure lipid, confirming that the protein increases the rate of the metastable to stable state transition.

The broad higher temperature component (peak 2) seen on heating for NFA-L-CBS and NFA-S-CBS in the presence of basic protein and Na^+ was not observed in the presence of K^+. However, it may occur under the sharp main transition. The temperature of this transition was not decreased by basic protein in the presence of K^+ as it was in Na^+ and this may prevent detection of the broad component.

The effects of basic protein and polylysine in 0.01 M Na^+ on the phase transition of HFA-S-CBS are shown in Fig. 6. HFA-S-CBS does not form a stable phase in 0.01 M Na^+ (on the time scale of the experiment) although it does at high concentrations (greater than 0.5 M) of K^+ [11]. Thus, only a single peak of similar T_m and ΔH on heating and cooling is observed for pure HFA-S-CBS (Fig. 6a,d and Table I). In the presence of 19% basic protein two endothermic transitions are present in the thermogram, one (peak 2) at a similar temperature as the pure lipid but broader and of reduced enthalpy, and the other (peak 1) at a lower temperature and of low enthalpy (Fig. 6b and Table I). The thermogram appeared very similar on cooling (Fig. 6e) unlike the case with NFA-L-CBS and NFA-S-CBS. The scans had a similar appearance at slower heating and cooling rates. This calorimetric behavior was not suggestive of metastable phase behavior or formation of the

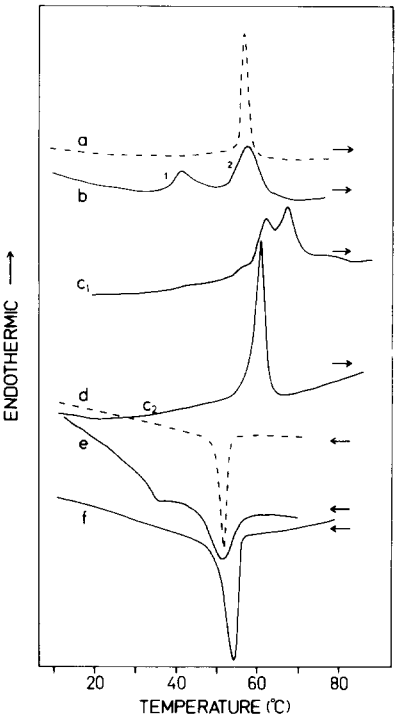

Fig. 6. DSC scans of HFA-S-CBS in 0.01 M Na^+ at heating ($a-c_2$) and cooling (d–f) rates of 10 deg. C/min. (a,d) lipid only; (b,e) 19 wt.% basic protein; (c,f) 30 wt.% polylysine; (c_1) first heating scan after preparation of the sample; (c_2) a typical reheating scan. Sensitivity settings in mcal/s were (a,d) 0.8; (b,e) 0.3; (c_1,c_2,f) 0.3. All other details as in caption to Fig. 1.

stable state, indicating that the lower temperature peak seen on heating and cooling was caused by a perturbing effect of the protein on a portion of the lipid similar to its effect on other acidic lipids [1,3]. The spectrum of 16-S-SL at 9°C in the complex of HFA-S-CBS with basic protein (Fig. 7C) was more immobilized than in the metastable state of the pure lipid (Fig. 7A), but not as much as in the complex of basic protein with non-hydroxy fatty acid forms of the lipids as indicated by the T_{max} values in Table III. Furthermore, at 30°C the spectrum was anisotropic (Fig. 7D), characteristic of the metastable state of the pure lipid (Fig. 7B), and not like that in the stable state of the complex of basic protein with the non-hydroxy fatty acid forms shown in Fig. 3C. This supported the conclusion that HFA-S-CBS does not go into the stable state in the presence of basic protein.

In the presence of polylysine at least four com-

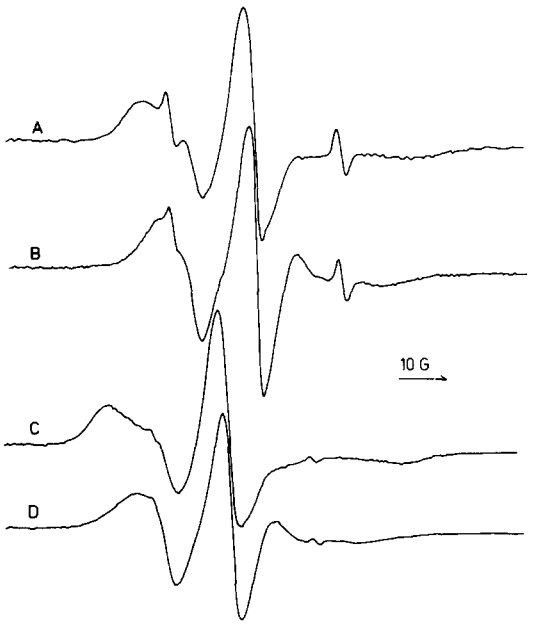

Fig. 7. ESR spectra of 16-S-SL in HFA-S-CBS in 0.01 M Na^+. A, no basic protein, 9°C; B, no basic protein, 30°C; C, 19% basic protein, 9°C; D, 19% basic protein, 30°C.

ponents were present in the thermogram on the first heating scan (Fig. $6c_1$). On the second and repeated heating scans, however, only one sharp peak occurred in the thermogram at 60.4°C (Fig. $6c_2$). The T_m and ΔH were similar on reheating and cooling (Fig. $6c_2$,d and Table I). This suggested that the transition in Fig. $6c_2$ is that of the metastable state of the complex, while the sample might have been partially in the stable state after preparation and before heating. Incubation of the sample at various temperatures at and close to 60.4°C, which causes other metastable forms of these lipids to go into their stable states [11], did not result in a higher melting state or a DSC scan resembling the first heating scan. These results suggested that although the complex of polylysine with HFA-S-CBS might occur in the stable state after preparation of the sample, it did not go back into the stable state on the time scale of the experiment, after melting.

The effect of basic protein on the calorimetric behavior of NFA-P-CBS resembled its effect on HFA-S-CBS. NFA-P-CBS goes into its stable state immediately on freezing from the liquid crystalline phase [11] so that only one peak with similar T_m and ΔH occurs on heating and cooling (Fig. 8a,d and Table I). (At 0.01 M Na^+, the peak on cooling is almost split into two components (Fig. 8d) but not at higher cation concentrations [10,11]. Basic protein decreased the enthalpy but has little effect on the temperature of the main transition on heating and cooling (Fig. 8b,e and Table I). It also caused the appearance of another broad peak at a lower temperature (Fig. 8b). On cooling, the temperature of this peak was decreased further and it was even broader. The spectra of 16-S-SL in the complex of basic protein with NFA-P-CBS were similar to the spectra in the complexes of the longer non-hydroxy fatty acid chain forms of cerebroside sulfate with basic protein, suggesting that the complex of basic protein with NFA-P-CBS also goes into a stable phase (see Table III for T_{max} values at 9°C). The main high temperature transition in Fig. 8b is probably that of the stable phase, while the lower temperature transition is probably that of a portion of the lipid which is too perturbed by the protein to go into the stable phase.

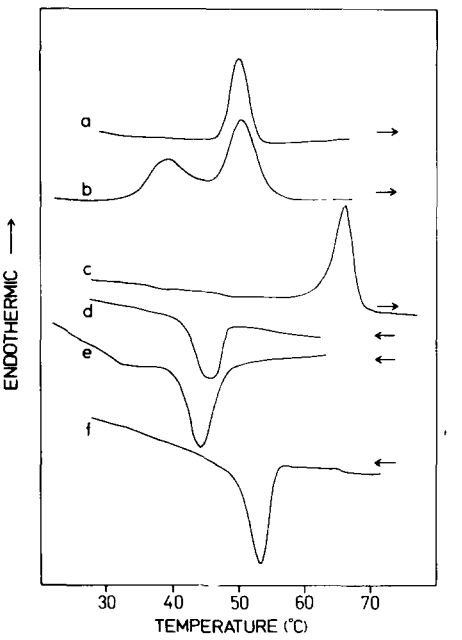

Fig. 8. DSC scans of NFA-P-CBS in 0.01 M Na^+ at heating (a–c) and cooling (d–f) rates of 10 deg. C/min. (a,d) lipid only; (b,e) 20 wt.% basic protein; (c,f) 30 wt.% polylysine. Sensitivity settings in mcal/s were (a,d) 1.0; (b,e) 0.3; (c,f) 0.5. Other details as in caption to Fig. 1.

Polylysine increased the T_m of NFA-P-CBS similarly to its effect on the other lipids (Fig. 8c). On cooling (Fig. 8f) the T_m was 8 deg. C below that on heating (Table I), indicating that the transition observed on heating in Fig. 8c was probably that of the stable state and that the sample refroze into the metastable state.

Basic protein had a similar effect on the calorimetric behavior of HFA-P-CBS as on NFA-P-CBS except that it decreased the T_m and broadened the transitions more. HFA-P-CBS only goes into the stable state at a high K^+ concentration but not at 0.01 M Na^+. The transitions shown in Fig. 9a,c are the melting and freezing transitions, respectively, of the metastable state of the pure lipid [10,11]. Basic protein broadened the main transition, decreased its enthalpy considerably and its temperature by two degrees (Fig. 9b and Table I). It also caused a very broad low enthalpy peak at lower temperatures. The scan appeared similar on cooling (Fig. 9d). The complex was not studied with 16-S-SL because of a shortage of lipid but it is probable that this lipid, like HFA-S-CBS, remains in the metastable state in the presence of basic protein.

Values of the motional parameter, τ_0, of 16-S-SL in the liquid-crystalline phase of all the samples are given in Table III. The motional parameter in the different cation or protein complexes of cerebroside sulfate increased in the order $Na^+ < K^+ < Ca^{2+}$ = basic protein. Thus, basic protein reduces the lipid motion in the liquid-crystalline phase.

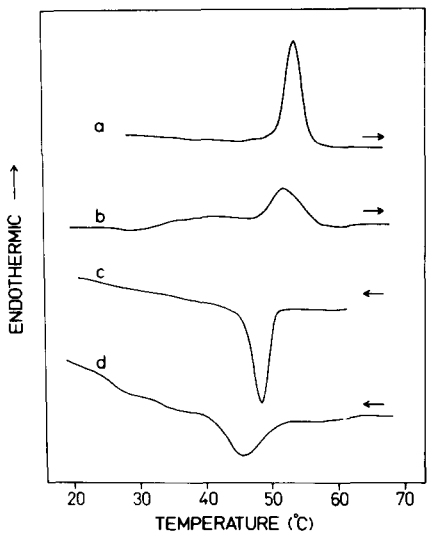

Fig. 9. DSC scans of HFA-P-CBS in 0.01 M Na^+ at heating (a,b) and cooling (c,d) rate of 10 deg. C/min. (a,c) lipid only; (b,d) 33 wt.% basic protein. Sensitivity settings in mcal/s were (a,c) 0.8; (b,d) 0.3. Other details as in caption to Fig. 1.

Discussion

The calorimetric behavior of complexes of basic protein with NFA-L-CBS and NFA-S-CBS indicated that they undergo a transition from a metastable to a stable state on cooling as does the pure lipid in the presence of certain cations. The decreased enthalpy of the stable state to liquid-crystalline phase transition and the increased solubility of the fatty acid spin label in the BP-CBS complexes relative to the pure lipid indicates that basic protein disorders the stable state to some extent. The spin label was more immobilized in the stable state than in the metastable state, and thus the two states could be distinguished on the basis of their ESR spectra as well as their calorimetric behavior. If the appearance of a powder spectrum for 16-S-SL can be taken as diagnostic of the stable state found in the presence of basic protein, then it can be concluded that basic protein also induces the same kind of stable state in NFA-P-CBS but not HFA-S-CBS.

Basic protein increases the rate of formation of the stable state of the non-hydroxy fatty acid forms of cerebroside sulfate, at least at low concentrations. This is indicated by the greater enthalpy on cooling of the metastable to stable state transition (peak 2) of the basic protein complex, relative to the pure lipid at similar cooling rates. However, at higher basic protein concentrations some of the basic protein sample remains in the metastable state, as is evident from the broad, higher temperature peak (peak 2 in Fig. 1b and 4c) remaining even after cooling to a low temperature, which converts most of the sample to the stable state. Thus, high concentrations of basic protein may inhibit or prevent formation of the stable state. At low protein concentrations the T_m of the liquid crystalline to metastable state transition is a few degrees higher than that of the pure lipid but decreases with increase in protein concentration. Basic protein decreases or has no effect on the T_m

of the stable state to liquid crystalline phase transition. The effect on ΔH of each state can not be determined, since it is not known what percentage of the lipid is in each state.

The hydroxy fatty acid forms probably do not go into the stable state in the presence of basic protein. Even in the absence of basic protein the hydroxy fatty acid forms do not go into the stable state as readily as the non-hydroxy fatty acid forms. Less protein binds to the hydroxy fatty acid than to the non-hydroxy fatty acid forms and only a portion of the lipid is perturbed as indicated by the appearance of two peaks in the thermogram, one at the same temperature as the pure lipid and one at a lower temperature. This contrasts with the behavior of acidic phospholipids such as phosphatidylglycerol, phosphatidic acid and phosphatidylserine which can be converted entirely to a lower melting form at high protein concentrations.

The calorimetric behavior of complexes of polylysine with NFA-L-CBS, NFA-S-CBS and NFA-P-CBS also clearly indicate that a stable state is formed. The stable state of polylysine with HFA-S-CBS which occurs after preparation of the sample does not reform once the sample has been melted.

The T_m of the stable state of the complex of polylysine with the non-hydroxy fatty acid forms of cerebroside sulfate is 15–16 deg. C above that of the pure lipid in the presence of 0.01 M Na^+ and a few degrees greater than that in the presence of 2 M K^+. The T_m of the metastable phase of the polylysine complexes is 7–8 deg. C above that of the pure lipid in the presence of 0.01 M Na^+. This large increase in T_m may be caused by more efficient shielding of the charge of cerebroside sulfate by polylysine and/or a cross-linking effect causing closer packing of the lipid. Polylysine also increases the T_m of acidic phospholipids [1,4]. It has less effect on the T_m of the stable and metastable phases of HFA-S-CBS than NFA-S-CBS. This is probably because the hydroxy fatty acid form packs more closely in the absence of polylysine than the non-hydroxy fatty acid form as a result of its increased intermolecular hydrogen bonding. Polylysine decreases the rate of formation of the stable state of NFA-L-CBS but not of the shorter chain length forms. The rate of formation of the stable state in the presence of polylysine decreases with increase in chain length in the order palmitoyl > stearoyl > lignoceroyl form.

It is clear that basic protein does not have as great an effect on the T_m and ΔH of cerebroside sulfate as of acidic phospholipids, indicating that it does not perturb the lipid packing as much. Its effect on the phase transition and amount bound decrease as the chain length increases. The high degree of order of the stable state of the long chain forms of cerebroside sulfate may inhibit binding of the protein and its perturbing effect, once bound. This is attributed to the intermolecular hydrogen bonding interactions of this lipid, mediated by the galactose hydroxyls, hydroxyl groups on the fatty acid and sphingosine chains, and the amide moiety, which cause resistance to lateral separation and expansion of the lipids. Basic protein was found to increase the surface pressure of monolayers of natural cerebroside sulfate [15,16] suggesting that it had a perturbing effect which would have expanded the monolayer if the surface area had not been kept constant. However, the effect was much greater at low initial surface pressures than at high ones [16], when intermolecular hydrogen bonding is more likely to occur. These interactions are probably weaker than in cerebroside as a result of the negatively charged sulfate which has a repulsive effect and lowers the T_m of cerebroside sulfate relative to cerebroside [10]. However, they should be strengthened after electrostatic binding of basic residues of basic protein to the sulfate. These results help to support the conclusion that the lipid head-group dependence of the perturbing effect of basic protein on the bilayer is a consequence of the presence or absence of intermolecular hydrogen bonding interactions in the lipids [3,5,7].

On the other hand, in spite of its relatively small perturbing effect on cerebroside sulfate, basic protein does not increase the T_m relative to that of the pure lipid, unlike polylysine. This suggests that, although basic protein is also bound electrostatically to the sulfate, its small perturbing effect is sufficient to counteract the ordering effect of charge neutralization. The small perturbing effect which basic protein has on cerebroside sulfate would cause some lateral expansion of the lipids, explaining the increased solubility of the spin label probe in the stable state. It would also account for

the increased rate of formation of the stable state in the presence of basic protein if the mechanism is by interdigitation of the fatty acid chains. Interdigitation has not yet been demonstrated to occur for any sphingolipids, most of which undergo metastable phase behavior. However, a somewhat different form of interdigitation has been demonstrated by X-ray diffraction to occur in an unequal chain length form of phosphatidylcholine, 1-stearoyl-2-caproyl-*sn*-glycero-3-phosphocholine [17,18]. There is steric resistance to interdigitation and some expansion and loosening up of the bilayer should decrease this resistance. Rotational motion allowing rearrangement of the lipids should also be necessary for a transition from a noninterdigitated metastable state to an interdigitated stable state; divalent cations, the polyvalent cation polylysine, or the fatty acid hydroxyl group, which inhibit the transition to the stable state, cause increased cross-linking of the lipid and would be expected to inhibit lipid rearrangement and interdigitation. Therefore, the increased rate of formation of the stable state caused by basic protein and the decreased rate caused by polylysine are consistent with the mechanism of interdigitation.

It cannot yet be concluded whether a structure like that of the stable state might occur in myelin. The fact that it can occur in the presence of myelin basic protein shows that this protein at least would not prevent its formation in myelin. However, the effects of other lipids and proteins in myelin on formation of the stable state must also be determined.

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