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## LIPID-PROTEIN INTERACTIONS IN MODEL MEMBRANES FROM BOVINE BRAIN WHITE MATTER

### AN ESR SPIN LABEL AND ELECTRON MICROSCOPY STUDY

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#### SUMMARY

Lipid-protein model membranes, prepared from bovine brain white matter and containing all the lipids and Folch-Lees proteolipids, have been studied in macroscopically oriented multibilayers. To examine the lipid environment the membranes were spin labeled with the cholestane spin label (3-spiro(2'-(*N*-oxyl-4',4'-dimethyloxazolidine))5 $\alpha$ -cholestane) and a fatty acid spin label (4',4'-dimethyloxazolidine-*N*-oxyl derivative of 5-ketostearic acid). The ESR spectra exhibit two components arising from fairly well oriented and completely unoriented lipids. Up to a temperature of 55 °C the amount of oriented lipids is almost constant, being about 35 %. At higher temperatures this percentage drops rapidly to zero. It is shown that the presence of unoriented lipids arises mainly from disrupted areas in the lipid bilayer structure. This is confirmed by electron microscopy and from an analysis of the temperature dependence of the order parameters of the spin labels. The presence of locally disrupted lipid parts in the bilayer is discussed in relation to the interaction of the brain white matter lipids with Folch-Lees protein.

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#### INTRODUCTION

Lipid-protein interactions play an important role in the organization and functional properties of biological membranes [1, 2]. In general, biological membranes contain a large variety of lipids and proteins, which renders it very difficult to study the nature of lipid-protein interaction in detail. To overcome these difficulties it is a logical step to investigate the lipid-protein interaction in model systems containing well-defined lipids and proteins. A number of lipid-protein model systems have been studied in this way during recent years, using X-ray diffraction [3, 4], nuclear magnetic resonance spectroscopy [5, 6], electron spin resonance (ESR) spin labeling [7–14], and surface tension measurements in monolayers [15, 16].

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Bovine brain white matter is a suitable system to study lipid-protein interactions, because the protein and lipid compositions are well known [17, 18]. Bovine brain white matter contains about 20 % protein [18]. The Folch-Lees protein [19, 20] accounts for approx. 60 % of the total protein and much of the remainder is the basic protein [17]. The protein content of brain white matter is low compared with biological membranes, which contain about 60 % protein [21]. Furthermore, the white matter proteins have no enzymatic activity [21]. However, these proteins have structural functions in myelin [22]; hence a study of the interaction of these proteins with lipids may give information that is relevant to biological membranes. The most abundant lipids are cholesterol, cerebrosides, phosphatidylethanolamine and phosphatidylcholine [18]. In addition, a relatively high content of charged lipids is present, such as cerebroside sulfate (sulfatides), phosphatidylserine and triphosphoinositides [18].

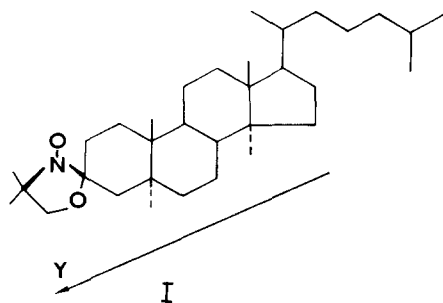
In this work chloroform/methanol-soluble lipid-protein material from bovine brain white matter was studied, which contains essentially all the lipids and Folch-Lees proteolipids [20]. This material forms bilayer structures which can be macroscopically oriented between glass plates. The ESR spin label technique was used to study the oriented multibilayers with the cholestane spin label I and the fatty acid spin label II. ESR spectra of these spin labels incorporated into the lipid-protein model membranes provide information about structural and dynamic properties of the lipids in the membranes. In addition, the lipid-protein material was studied by electron microscopy. Our results strongly suggest that the Folch-Lees protein has a disturbing effect on the local lipid organization, without disrupting the macroscopic bilayer organization. A preliminary report of this work has been published as part of a thesis [23].

## MATERIALS AND METHODS

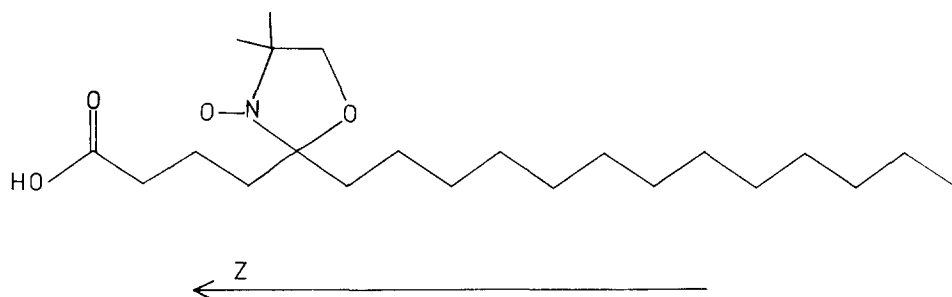
### *Preparation of spin-labeled membranes*

Bovine brain white matter was extracted by the two phase KCl method [20]. The chloroform/methanol phase was filtered and after centrifugation a clear, slightly yellow solution was obtained. The protein content in this solution was determined by measuring the absorption at 278 nm [20], giving a value of 11.6 mg protein per 100 mg dry lipid-protein material.

Two preparations were made from this solution, one containing the cholestane spin label I (3-spiro(2'-(*N*-oxyl-4',4'-dimethyloxazolidine))-5 $\alpha$ -cholestane) and the



Formule I



II

## Formule II

other containing the fatty acid spin label II (4',4'-dimethyloxazolidine-*N*-oxyl derivative of 5-ketostearic acid). Spin label I was synthesized by the method of Keana et al. [24]. Spin label II was obtained from Syva Associates. Both spin labels were added in a concentration of 0.50 mg per 100 mg dry weight (about 1 mol%). The solvents were evaporated and the membrane material was stored at room temperature in a dessicator above distilled water, to maintain the samples hydrated.

*Orientation of the membranes*

Small amounts of spin-labeled membranes were oriented between glass plates as described previously [25, 26]. The macroscopic ordering was checked with a polarizing microscope [27]. The ordering could be increased by equilibrating the oriented multibilayers between the glass plates for about 24 h in the dessicator above distilled water.

*ESR measurements*

ESR measurements were carried out with a stack of glass plates sealed in a quartz capsule above a 28 %  $\text{H}_2\text{SO}_4$  solution, which has a temperature-independent relative humidity of 80 % (calculated from the Landolt-Börnstein Tables). The quartz capsule was rotated in the cavity so that the normal to the plane of the glass plates (or optical axis) was either parallel ( $\theta = 0^\circ$ ) or perpendicular ( $\theta = 90^\circ$ ) to the external magnetic field. ESR spectra were recorded on a Varian E3 X-band spectrometer, equipped with a temperature controller. The temperature in the capsule was measured with a copper-constantan thermocouple and is accurate to  $\pm 0.5^\circ\text{C}$ .

*Electron microscopy*

Samples of spin-labeled membranes were transferred to gold specimen holders and heated at the desired temperature for about 1 min. There after the samples were rapidly quenched in Freon-22. Specimens were freeze-broken on a Balzer machine and shadowed with a Pt-C electron gun. Replicas were cleaned with 40 % chromic acid. Electron micrographs were made on a Philips EM-200 at 80 kV.

## RESULTS

*Spectral analysis*

Some typical ESR spectra of spin labels I and II in the oriented membrane

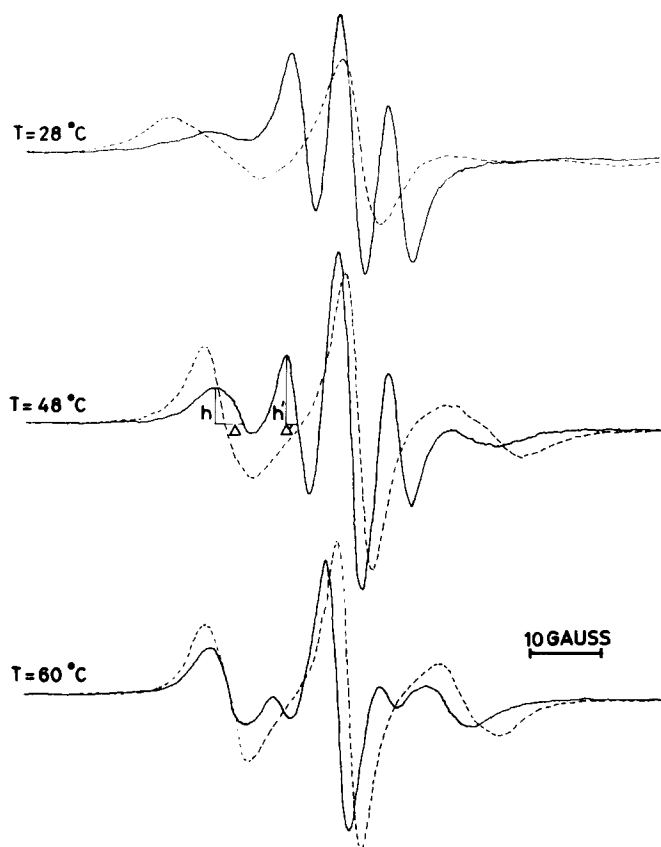


Fig. 1. ESR spectra of the cholestane spin label I in the lipid-protein model membranes from bovine brain white matter, macroscopically oriented between glass plates. In the solid line spectra the normal to the plane of the glass plates is parallel ( $\theta = 0^\circ$ ) to the external magnetic field. In the dotted spectra the normal is perpendicular ( $\theta = 90^\circ$ ) to the external magnetic field. The temperature is 28, 48 and 60 °C.  $\Delta$ ,  $\Delta'$  and  $h$ ,  $h'$  indicate the half-width and height of the low field peaks of the two spectral components (see text).

system at different temperatures are given in Figs. 1 and 2. At 28 °C the spectra exhibit a strong angular dependence, which arises from spin labels oriented in the membrane bilayers. At higher temperatures (above 60 °C) the angular dependence has almost disappeared, indicating that most of the lipids are in an unoriented state. To distinguish between these two states of the lipids, we will speak about “oriented” lipids when dealing with fairly well oriented lipid molecules, and “unoriented” lipids when the lipids are in a completely unoriented state.

At a temperature of 28 °C unoriented lipids are present also, as can be seen in the  $\theta = 0^\circ$  spectra of spin label I in Fig. 1. The spectral component with three sharp lines and a hyperfine splitting of 6.5 G arises from the oriented lipids, while the spectrum with broader lines, which is quite similar to the spectra at 60 °C, arises from unoriented lipids. In Fig. 1, it can be seen that the disappearance of the angular dependence is accompanied by the decrease of the spectral component arising from the

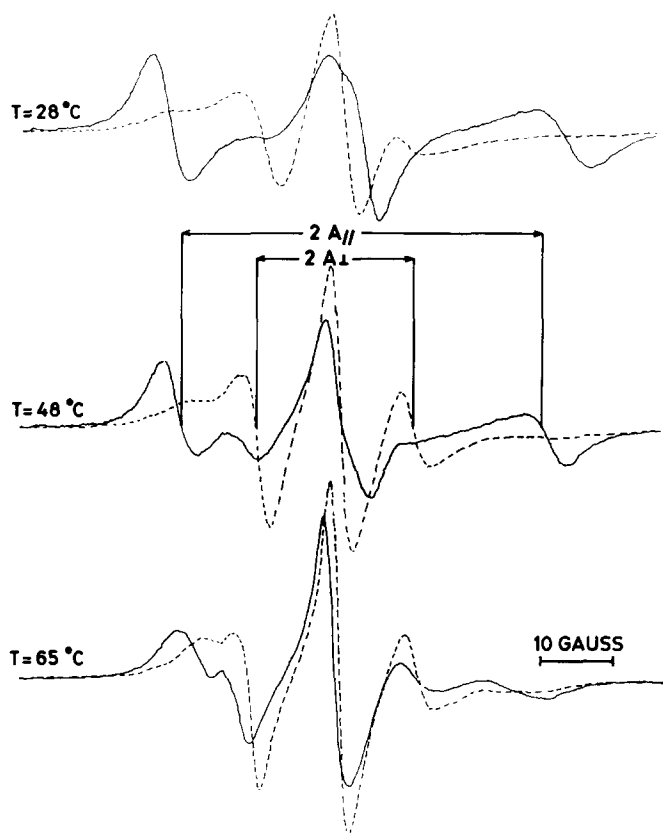


Fig. 2. ESR spectra of the fatty acid spin label II in the lipid-protein model membranes from bovine brain white matter, macroscopically oriented between glass plates. In the solid line spectra  $\theta = 0^\circ$  and in the dotted spectra  $\theta = 90^\circ$ . The temperature is 28, 48 and 65 °C.  $A_{||}$  and  $A_{\perp}$  are the hyperfine splittings in the  $\theta = 0^\circ$  and  $\theta = 90^\circ$  spectrum.

oriented lipids. The two spectral components can also be observed in the  $\theta = 90^\circ$  spectra of spin label II in Fig. 2, but here the components are not well resolved because the hyperfine splitting of the oriented lipids is larger and the spectral component arising from unoriented lipids has a more complicated line shape [14].

To quantitate the amount of oriented and unoriented lipids we used the ESR spectra of spin label I in Fig. 1. We will assume that spin label I is uniformly distributed over the whole system [14], so that the area under the absorption peaks of the two spectral components is proportional to the amount of oriented and unoriented lipids. For derivative lorentzian peaks this area is proportional to  $h\Delta^2$ , where  $\Delta$  is the half-line width and  $h$  the height of the peaks (see Fig. 1). For this calculation the low field peaks of the two spectral components were taken. Although the line shape of both spectral components may not be perfectly lorentzian, this calculation procedure is a reasonable way to obtain quantitative information about the distribution of spin label I. It must be emphasized, however, that this calculation gives only approximate values for the amount of oriented and unoriented lipids. The results of the calculation

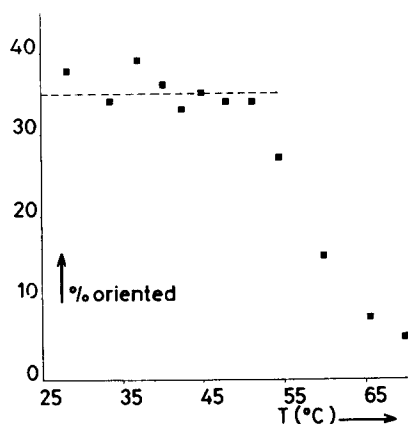


Fig. 3. Temperature dependence of the relative amount of oriented lipids ( $h' \cdot l'^2 / (h' \cdot l'^2 + h \cdot l^2)$ , see Fig. 1) in the macroscopically oriented lipid-protein model membranes.

are given in Fig. 3. The plot in Fig. 3 is completely reproducible and the temperature dependence is reversible.

As can be seen in Fig. 3, at room temperature about 35 % of the lipids is oriented. Up to 55 °C this percentage is almost constant, but drops rapidly above 55 °C. It is clear that, even at room temperature, a large part of the lipids (> 65 %) must be unoriented. There are two possible sources for the presence of unoriented lipids: (a) The unoriented lipids are present in randomly oriented membrane bilayers. This can be called macroscopic disordering, or (b) the unoriented lipid parts arise from local disturbances in the bilayers, giving rise to small unoriented lipid parts embedded in a bilayer structure.

With a polarizing microscope it is possible to distinguish between macroscopical unoriented lipids (type a) and well-oriented lipids [25–27]. The type b unoriented lipids will be indistinguishable from oriented lipids. When after the ESR experiments the samples between glass plates were studied under the polarizing microscope at room temperature, it was found that, except for the presence of macroscopically unoriented material at the edges of the glass plates (< 20 %), the lipid-protein material was macroscopically well-oriented. This means that, as a rough estimate, at room temperature about 45 % of the bilayer will be locally disrupted.

It will be clear that this estimate depends largely on the assumption that spin label I is uniformly distributed over the oriented and unoriented parts of the lipid bilayer. When the spin label dissolves preferentially in the unoriented part this estimate will be too high, while in the opposite case this estimate will be too low.

### *Electron micrographs*

To observe the effect of temperature on the local ordering of the bilayers, electron micrographs have been made of membrane samples quenched at 25 °C and 70 °C (see Fig. 4). An almost regular bilayer structure is observed at 25 °C, but at 70 °C most of this structure has disappeared. Since the effect of macroscopical disordering extends over much larger areas, as shown by the electron micrographs, we must conclude that the rapid decrease of the amount of oriented lipids above 55 °C in Fig. 3 is a local disrupting effect in the membrane bilayers.

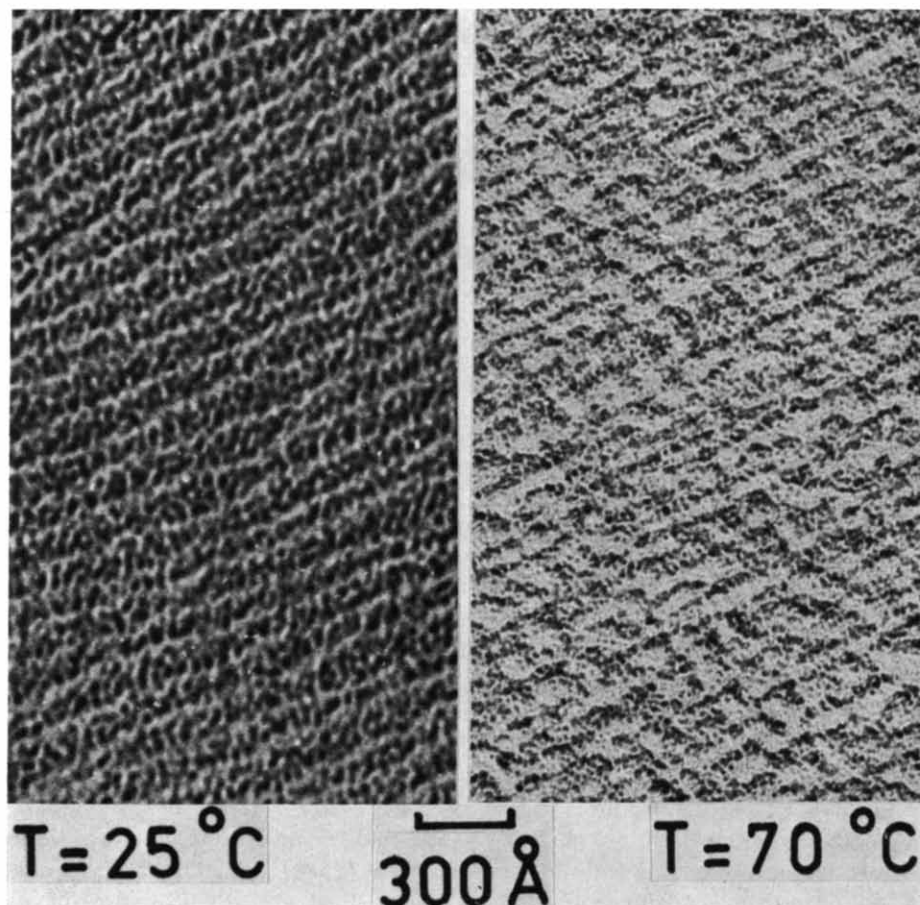


Fig. 4. Electron micrographs of the lipid-protein model membranes at 25 and 70 °C.

A qualitative comparison of the amount of locally disrupted lipids in the membrane bilayers calculated from the ESR spectra and the electron micrographs is not possible. The resolving power of the electron microscope is about 20 Å, making it difficult to calculate the amount of locally disrupted lipids from the electron micrographs.

It is interesting to compare our electron micrographs with the results of Mateu et al. [4]. They found an asymmetry in model membranes of the basic protein and acidic lipids, giving rise to a repeat distance of 180 Å in X-ray diffraction patterns, similar to myelin. By electron microscopy they showed that the bilayers were coupled two by two. In our model system, which contains the Folch-Lees protein, the thickness of the bilayers, as determined from the electron micrographs, is 60–70 Å. Therefore, it can be concluded that both myelin proteins influence the structure of the model membrane system in a different way. This corresponds well with the surface tension measurements of London et al. [15, 16], who found that both myelin proteins have a different lipid affinity. Basic protein is mainly bound by acidic lipids, whilst the Folch-Lees protein has a much broader lipid affinity pattern.

### Order parameters

The degree of ordering of the z-axis,  $S_z$ , of a nitroxide spin label can be expressed as [25, 26]

$$S_z = \frac{A_{\parallel} - A_{\perp}}{A_z - A_x}, \quad (1)$$

where  $A_{\parallel}$  is the hyperfine splitting of the ESR spectrum of the spectral component arising from the oriented lipids at  $\theta = 0^\circ$  and  $A_{\perp}$  is the splitting at  $\theta = 90^\circ$ .  $A_x$ ,  $A_y$  and  $A_z$  are the components of the hyperfine tensor, which is assumed to be axially symmetric, i.e.  $A_x = A_y$  [25]. In general,  $A_x$  and  $A_z$  can be used in Eqn. 1 after correcting for the polarity of the environment in which the spin label is located [26]. The order parameter  $S_z$  is a measure for the mean square deviation of the z-axis of the nitroxide spin label with respect to the normal to the bilayer. A value of one is found for the order parameter in the case of parallel orientation, while an isotropic motion gives a value of zero. In the case of perpendicular orientation the order parameter is  $-\frac{1}{2}$ .

When the fatty acid spin label II is considered, the z-axis of the nitroxide group points along the long chain axis and Eqn. 1 immediately gives the order parameter  $S$  of the long fatty acid chain at the 5-position. In most cases  $A_{\parallel}$  and  $A_{\perp}$  are obtained from the base line crossings of the peaks in the ESR spectra as indicated in Fig. 2. In the spectra below  $40^\circ\text{C}$  determination of  $A_{\perp}$  is difficult, because the lines in the  $\theta = 90^\circ$  spectra are broadened. In this case  $A_{\perp}$  is calculated from  $a_{\text{iso}}$  and  $A_{\parallel}$ , using the relationship

$$a_{\text{iso}} = \frac{1}{3}(A_{\parallel} + 2A_{\perp}). \quad (2)$$

$a_{\text{iso}}$  is the isotropic value of the hyperfine tensor, calculated with Eqn. 2 from the spectra where both  $A_{\parallel}$  and  $A_{\perp}$  could be obtained and is constant within the limits of experimental error. This indicates also that the error in the determination of  $A_{\parallel}$  and  $A_{\perp}$  in Fig. 2 is small. The same procedure is used for the ESR spectra above  $55^\circ\text{C}$ , where the base line crossings of the outer peaks are difficult to determine.

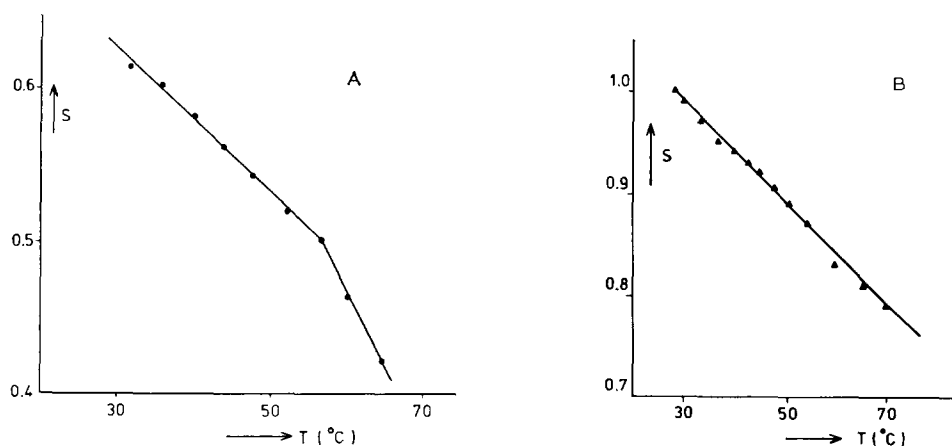


Fig. 5. Temperature dependence of the order parameter  $S$  of the fatty acid spin label II (A) and the cholestane spin label I (B).



The temperature dependence of  $S$  is given in Fig. 5A. The points in Fig. 5A lie on two straight lines, which intersect at 56 °C, corresponding approximately with the temperature where the amount of oriented lipids sharply decreases (see Fig. 3).

It is also possible to determine the order parameter  $S$  of the long axis of the steroid nucleus of the cholestane spin label I. However, in this case the y-axis of the nitroxide group coincides with the long axis, and Eqn. 1 must be modified to calculate  $S$ . Making use of the observation that the order matrix of spin label I is almost axially symmetric [26], i.e.  $S_z = S_x = -\frac{1}{2}S_y$ , we obtain from Eqn. 1:

$$S = S_y = -2 \frac{A_{\parallel} - A_{\perp}}{A_z - A_x}. \quad (3)$$

Since the spectral components of the oriented and unoriented lipid parts at  $\theta = 90^\circ$  are not well resolved (see Fig. 1), the hyperfine splitting  $A_{\perp}$  is difficult to determine. However, using Eqn. 2 the following result is obtained:

$$S = 3 \frac{a_{\text{iso}} - A_{\parallel}}{A_z - A_x}. \quad (4)$$

This equation is very useful because  $A_{\parallel}$  can be accurately determined, even when the spectral component arising from the oriented lipids is small.  $a_{\text{iso}}$  is determined from the hyperfine splitting of the spectral component of the unoriented lipids and is nearly constant in all the spectra of spin label I, giving  $a_{\text{iso}} = 15.0$  G. Strictly speaking  $a_{\text{iso}}$  may not be determined in this way, because the spectral component of the unoriented lipids is a powder-like ESR spectrum and not an isotropic spectrum. However, the constant value of  $a_{\text{iso}}$  of 15.0 G is in good agreement with other work [26], justifying our procedure.

In Fig. 5B the variation of the order parameter  $S$  of spin label I with temperature is given. Note that  $S$  is now determined only from the spectral component arising from the oriented lipids. Hence Fig. 5B gives the ordering of lipid molecules in the oriented parts of the bilayer. In contrast with Fig. 5A, a straight line is found, indicating the absence of a sharp phase transition in the lipids in the oriented parts of the bilayer.

The value of the order parameter  $S$  of the cholestane spin label I is nearly 1 (see Fig. 5B), indicating that the long molecular axis of spin label I is almost perpendicular to the bilayer surface. This high value of  $S$  also suggests that the lipid molecules in the oriented bilayer parts are strongly packed and have a high degree of order. The values of the order parameter of the fatty acid spin label II (Fig. 5A) are lower than the values obtained with the cholestane spin label I (Fig. 5B), because internal motions in the flexible fatty acid chain reduce the ordering of the chain.

### *Molecular motion*

From the  $\theta = 0^\circ$  spectra in Fig. 1 it is apparent that the line width of the spectral component arising from the unoriented lipids decreases when the temperature is raised. An Arrhenius plot of the linewidth  $\Delta$  of the low field peak against  $1/T$  is given in Fig. 6.  $\Delta$  is a direct measure of the mobility of the cholestane spin label I in the unoriented lipid part. In Fig. 6 the points lie on two straight lines which inter-

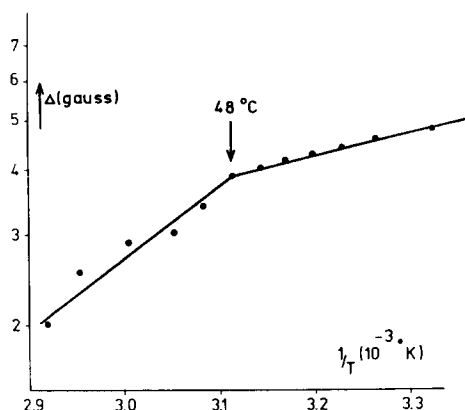


Fig. 6. Arrhenius plot of the line width  $\Delta$  of the low field peak of the spectral component of the cholestane spin label I arising from the unoriented lipids.

sect at a temperature of  $48^\circ\text{C}$ . This temperature again corresponds with the point in Fig. 3 where the amount of oriented lipids sharply decreases.

The activation energy below  $48^\circ\text{C}$  is  $3.6 \pm 1.3$  kcal/mol and a value of  $11.3 \pm 0.9$  kcal/mol is found above this temperature. The change of activation energy at  $48^\circ\text{C}$  is consistent with an alteration of the lipid composition of the unoriented part of the lipids at this temperature. This also implies that below  $48^\circ\text{C}$  the composition of the oriented and unoriented lipid parts will be different.

In the calculation of the amount of oriented lipids (Fig. 3), it was assumed that spin label I is uniformly distributed over the oriented and unoriented lipid parts. In this complicated lipid system, where oriented and unoriented lipid parts have different compositions, this assumption may not be entirely valid. Therefore, the absolute error in the calculated amount of oriented lipids can be large, however, this effect will not influence the qualitative nature of Fig. 3.

## DISCUSSION

The cholestane spin label I and fatty acid spin label II used in this study provide information about the physical state of the lipids in the lipid-protein membranes from bovine brain white matter. By macroscopic orientation of the lipid-protein samples, two lipid phases could be identified. One part of the lipids is present in oriented bilayer structures, whereas the other part is present in unoriented, locally disrupted areas of the lipid bilayer. Since there is a marked decrease of the oriented bilayer parts above  $55^\circ\text{C}$  (see Fig. 3), we will confine our discussion firstly to the properties of the system below this temperature.

In contrast to our previous report [23], we are led to propose that the Folch-Lees protein is responsible for the presence of the unoriented lipid parts in the lipid-protein system. The Folch-Lees protein is present in a concentration of about 12%. It is known that this protein binds charged lipids very tightly [15, 20], increasing the hydrophobic character of the protein surface. From the molecular weight of the Folch-Lees protein [20], it can be estimated that this lipid-protein aggregate has a

diameter of about 60 Å. Such a proteolipid is difficult to fit in the lipid bilayer, without causing a local disruption of the bilayer structure. This conclusion is in agreement with work of Butler [28], who studied the effect of the Folch-Lees protein on lipid structure.

From the ESR spectra in Fig. 1, where the oriented and unoriented lipid parts can be clearly identified, no indication was found for strongly bound lipids in the model membrane system, probably because the amount of tightly bound lipids in the lipid-protein aggregates is too small to give rise to a detectable ESR spectrum. However, taking into account that only charged lipids are present in the tightly bound region, it will not be surprising that the cholestane spin label I is excluded from this lipid region.

Above 55 °C, the amount of oriented lipid bilayers in the sample abruptly decreases (see Fig. 3). At about the same temperature, effects are shown by the order parameter of spin label II (Fig. 5A), as well as by the temperature dependence of the line width of spin label I in the unoriented lipid part (Fig. 6). The effects displayed in Figs. 5A and 6 are due to a change of lipid composition in the unoriented lipid part, when the oriented bilayer parts decrease in size. We are led to conclude that the lipid-protein complexes, consisting of the Folch-Lees protein, tightly bound charged lipids and the unoriented lipids, are embedded in the bilayer structure. This situation is schematically depicted in Fig. 7.

In Fig. 7, the organization of the unoriented and tightly bound lipids in the lipid-protein complex is not shown in detail, because it cannot be deduced from the available data. The lipid bilayer is of the fluid type and its thickness is about 50 Å, as estimated from studies with lipid model systems [29]. The thickness of the lipid-protein complex (80 Å) is calculated by assuming that the thickness as found from the electron micrographs (60–70 Å) is the average of the thickness of the lipid bilayer and the lipid-protein complex. Since at room temperature the amount of lipid bilayer parts and unoriented lipids is almost equal, the lipid-protein interactions will extend to large distances in the bilayer plane.

It is interesting to note that at room temperature our lipid-protein model system is in an unstable state, because a part of the lipid bilayers is locally disrupted. To our knowledge this has not been found with myelin, which contains the basic protein as well as the Folch-Lees protein. This indicates that the basic protein must influence the interactions between the lipids and the Folch-Lees protein, causing a stabilization of the bilayer structure, in agreement with the conclusions of London et al. [22].

Finally we remark that our results may be of interest for the study of multiple sclerosis. This disease attacks the basic protein and causes a disruption of the myelin

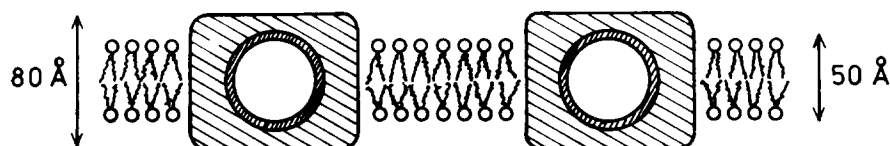


Fig. 7. A cross-sectional view of a model for the lipid-protein membranes from bovine brain white matter. The rectangle indicates the lipid-protein complex, consisting of the Folch-Lees protein (open circle), tightly bound charged lipids (densely cross-hatched) and the unoriented lipids (lightly cross-hatched), embedded in the fluid lipid bilayer.

sheet around the nerve fibers [22, 30], giving a situation similar to that studied in our lipid-protein model membranes, that do not contain the basic protein.

## CONCLUSIONS

Lipid-protein model membranes, prepared from bovine brain white matter and containing the Folch-Lees protein and all the lipids, consist of lipid-protein complexes embedded in lipid bilayers. The interaction between the lipids and the Folch-Lees protein in the complexes is in such a way that these lipids are in an un-oriented state.

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