

MOLECULAR DYNAMICS OF CEREBROSIDE-CHOLESTEROL AND SPHINGOMYELIN-CHOLESTEROL INTERACTIONS: IMPLICATIONS FOR MYELIN MEMBRANE STRUCTURE

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1. Introduction

Cholesterol, cerebroside and sphingomyelin are major components of myelin [1]. The study of the molecular dynamics of cerebroside (galactosyl derivatives of *N*-acyl sphingosine) and sphingomyelin (phosphorylcholine derivatives of *N*-acyl sphingosine) interactions with cholesterol should thus help our understanding of myelin membrane structure. Some preliminary studies of sphingomyelin-cholesterol interactions have recently been reported, [2, 3]. Here we attempt extension and clarification of some of the results obtained, together with some new information on cerebroside-cholesterol interactions. We have used the techniques of differential scanning calorimetry (DSC) and nuclear magnetic resonance spectroscopy (NMR) to study the mobility of the hydrocarbon chains and headgroups in these lipid systems in the presence and absence of cholesterol, as a function of temperature.

2. Experimental

Cholesterol was purchased from Fluka, Buchs, and was recrystallised twice from ethanol. Ox brain cerebroside were purchased from Koch-Light Ltd., Colnbrook, Bucks., (batch no. 44042), and ox brain sphingomyelin from Koch-Light, (batch no. 40296) and Schwartz-Mann, Orangeburg, New York, (batch no. W3923). All lipids were pure by TLC.

NMR spectra were obtained on a Varian HR-220 spectrometer. DSC thermograms were obtained on a Perkin-Elmer DSC-1B differential scanning calorimeter

at a heating rate of $8^{\circ}\text{K min}^{-1}$. Samples were prepared as described previously [2, 4]. For NMR spectroscopy the lipids were 20 wt. % hand-dispersions in 99.7% D_2O (Prochem Ltd., Croydon).

3. Results and discussion

Fig. 1 shows the DSC traces obtained on a) sphingomyelin, b) sphingomyelin-cholesterol (1:1), c) cerebroside, and d) cerebroside-cholesterol (1:1). All lipids were in excess water (5 mg lipid in $10\ \mu\text{l H}_2\text{O}$). Figs. 2 and 3 show the 220 MHz proton NMR spectra ob-

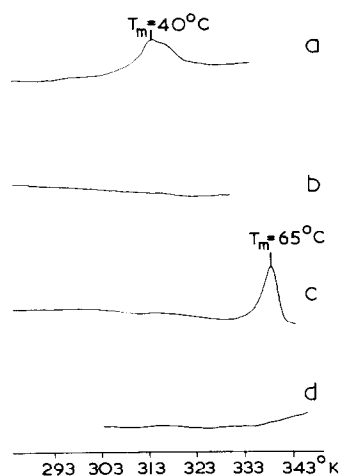


Fig. 1. Differential scanning calorimetry traces of: a) ox brain sphingomyelin; b) sphingomyelin-cholesterol (1:1); c) cerebroside; d) cerebroside-cholesterol (1:1); all in excess water.

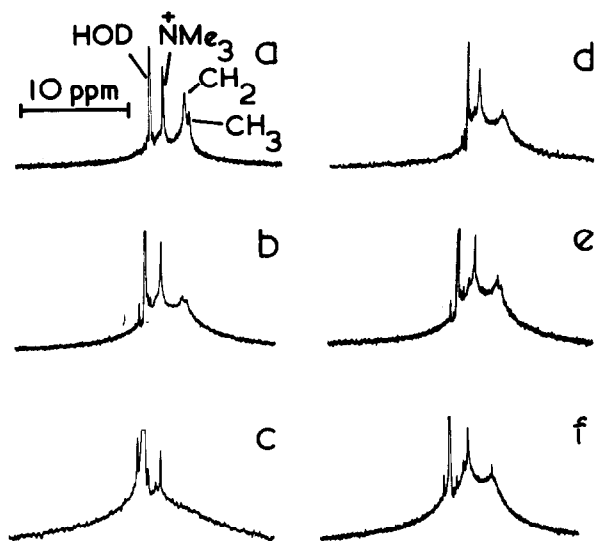


Fig. 2. 220 MHz ^1H -NMR spectra of 20 wt. % dispersions in D_2O of: a) sphingomyelin, 60°C ; b) 40°C ; c) 20°C ; d) sphingomyelin-cholesterol (1:1), 60°C ; e) 40°C ; f) 20°C .

tained from the above systems as a function of temperature.

The DSC heating curve of sphingomyelin (identical results were obtained on material from both sources) in excess water shows (fig. 1a) that a thermal transition occurs in the temperature range $32^\circ\text{C}\rightarrow 48^\circ\text{C}$, and has a maximum rate of melting (T_m) of 40°C . This corresponds to a gel \rightarrow liquid crystal phase transition of the hydrocarbon chains, and is consistent with the X-ray data of Reiss-Husson [5]. Addition of equimolar quantities of cholesterol to the sphingomyelin removes the thermal phase transition (fig. 1b). We suggest that this effect is quite analogous to the known behaviour of lecithin-cholesterol systems: above the thermal phase transition temperature range the chains are 'immobilised' by the cholesterol, and below the transition they are (predominantly [6]) 'fluidised' [7, 8]. A state of intermediate fluidity is produced by the cholesterol. This is borne out by the 220 MHz proton NMR results, fig. 2.

At 60°C the sphingomyelin is liquid crystalline, and a very well resolved spectrum is obtained, from a hand dispersed sample. Both the choline $-\text{NMe}_3$ and chain CH_2 and CH_3 groups are apparent (fig. 2a). The choline $-\text{NMe}_3$ group is highly mobile, as has been previously noted for the choline $-\text{NMe}_3$ group in lecithin in its

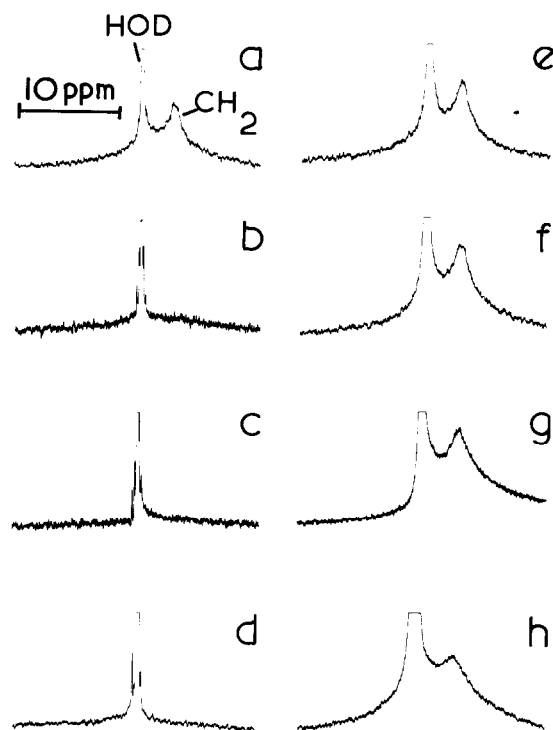


Fig. 3. 220 MHz ^1H -NMR spectra of 20 wt. % dispersions in D_2O of: a) cerebroside, 80°C ; b) 60°C ; c) 40°C ; d) 20°C ; e) cerebroside-cholesterol (1:1), 80°C ; f) 60°C ; g) 40°C ; h) 20°C .

smectic liquid crystalline phase in H_2O , using pulsed NMR and ^{13}C Fourier transform NMR [9, 10].

On the phase transition, at 40°C (fig. 2b), there is a decrease in the intensity and a broadening of the chain proton signals. On such a broad thermal phase transition both gel and liquid crystalline regions are present (due to the wide variety of N -acyl groups present [11]), and on the sweep width employed (5000 Hz), only the more mobile liquid crystalline regions will be resolved [12].

At 20°C (fig. 2c) it is clear that the lipid chains are relatively immobile (in a gel state [5]), since no chain signal is observed. (The small signal at the $-\text{NMe}_3$ resonance frequency may represent a minor choline-containing water soluble impurity). This is consistent with our earlier ESR results [2].

On addition of cholesterol to sphingomyelin above the temperature range of the phase transition, there is a dramatic broadening (fig. 2d) of the CH_2 signal of the

acyl chains, indicating a great restriction in their motional freedom. At 40°C (fig. 2e) the spectrum is rather similar to the 40°C sphingomyelin spectrum (fig. 2b), and corresponds to only a limited number of mobile methylene groups, probably near the methyl terminal ends of the *N*-acyl chains, many of the methylene groups being greatly immobilised by the rigid cholesterol ring structure [6]. Below the range of the sphingomyelin transition, at 20°C (fig. 2f), the spectrum of sphingomyelin-cholesterol (1:1) is rather similar to that obtained at 40°C and 60°C. This is in agreement with the DSC data, i.e. an 'intermediate fluid' state is formed which is relatively temperature insensitive over this range. This interpretation of a 'fluidisation' of sphingomyelin by cholesterol at 20°C is in good agreement with our previous work [2].

Long et al. [3] have confirmed our result of a fluid hydrocarbon chain region in sphingomyelin-cholesterol (1:1) in excess water at 20°C, using the spin-label 12-spiro-(2'-(*N*-oxyl-4', 4'-dimethyl oxazolidine))-stearic acid, but using the spin-label 3-spiro-(2'-(*N*-oxyl-4', 4'-dimethyl oxazolidine))-cholestane, have suggested that the presence of 50 mole % cholesterol causes a gel→liquid crystal phase transition to occur at 20°C, and that the mean transition temperature from a rigid to a fluid bilayer structure occurs at $32 \pm 0.5^\circ\text{C}$. Our calorimetric and NMR spectroscopic data are inconsistent with these suggestions.

These authors furthermore suggest that sphingomyelin thin films, in either the dry or hydrated state, do not form oriented multibilayers. That sphingomyelin exists in multibilayers is shown by the work of Reiss-Husson [5]. The reason for their lack of orientation at 20°C ($a_{\perp} \approx a_{\parallel} \approx 32$ gauss) could be attributable to the difficulty of orienting a gel (see Hsia et al. [13]), or at 37°C ($a_{\perp} \approx a_{\parallel} \approx 16.8$ gauss) to the difficulty of orienting a mixed gel-liquid crystal system. In such mixed systems the precise location of the spin-label is also uncertain [14], since it could preferentially probe the more fluid (liquid crystalline) domains in the system. In addition, we might also reasonably expect the *steroidal* spin-label to greatly perturb its local environment by preventing chain crystallisation – as has been suggested for the parent steroid, cholesterol [2, 4, 6–8], and as has been observed for other spin-labels [15], thus making extrapolation from the spin-label mobility to the mobility of the bulk (heterogeneous) lipid, difficult. This suggestion of a perturbation effect

of the spin-label would be consistent with its rapid (≥ 73 MHz) tumbling in the sphingomyelin at 37°C, and still quite rapid (> 73 MHz) tumbling in the bulk solid at 20°C.

Addition of cholesterol at 1:1 ratios results in a totally liquid crystalline sample which, possessing different *rheological* properties, orients under the experimental conditions used. This behaviour has previously been observed by Hsia et al. [13], who found that dipalmitoyl lecithin could only be oriented in the presence of ~50 mole % cholesterol, i.e. when the lipid was in a liquid crystalline state [7].

Similar considerations may apply to the work of Butler et al. [16], who studied white matter lipids with the cholestane label.

With cerebroside, the DSC data show $T_m = 65^\circ\text{C}$ (in good agreement with Clowes et al. [17]), with the total transition range being ca. $59^\circ\text{C} - 67^\circ\text{C}$ (fig. 1c). Equimolar quantities of cholesterol remove the thermal transition (fig. 1d). The NMR data (fig. 3) are in full agreement with the conclusion that cholesterol causes formation of an intermediate fluid, liquid crystalline state. At 40°C the protons in the cholesterol-cerebroside system (fig. 3g) are appreciably more mobile than in the cerebroside gel or in crystalline cholesterol [18].

Since cerebroside does not possess a phosphate-containing headgroup, it may be that specific "complex" formation in myelin, between lipids, involving headgroup phosphate-cholesterol 3-OH hydrogen bonding, is not of major structural importance. Liquid crystal formation between cholesterol and a variety of simple compounds has been demonstrated by Lawrence [19]. The principle structural requirement of the second component is that it be an amphiphile with a chain length $\geq n\text{-C}_{12}$ (which corresponds to the total length of the cholesterol molecule and is necessary for optimal van der Waals stabilisation).

It thus appears that a major structural role of cholesterol in myelin may be to cause formation of a liquid crystalline lipid phase from an otherwise mixed gel-liquid crystal system [20]. The role of cholesterol (in mammalian systems) may thus be to regulate fluidity, and hence permeability. It is worth noting, however, that mixed gel-liquid crystal clusters may be quite common in lower organisms which specifically lack cholesterol, e.g. *Acholeplasma laidlawii* B and *Escherichia coli* [14, 21], since broad thermal phase transi-

tions have been shown to encompass the growth temperature [21–24].

An additional point which requires further study is the way in which cholesterol affects the rheological properties of lipids. The rheological properties of lipid–cholesterol systems may be particularly important for myelin membrane mechanical stability.

Acknowledgements

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