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A calorimetric study of the thermotropic behaviour of mixtures of brain cerebrosides with other brain lipids

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We have used a computer-controlled differential scanning calorimeter to determine the phases present in mixtures of the brain galactocerebrosides with other representative brain lipids. There are two types of brain galactocerebroside, those which possess an α-hydroxy substituent on the acyl chain (HFA) and those that do not (NFA). In the liquid crystalline state both cerebrosides were miscible with all the lipids studied, but in the gel state they were immiscible with cholesterol and the brain phosphatidylcholines. However, cholesterol mixtures in which the cholesterol mole fraction exceeded one third formed homogeneous metastable gel states on cooling from above the melting point of the cerebroside. Relaxation to the stable two phase state took place slowly over several hours. The solubilities of the galactocerebrosides in the other main brain sphingolipid, sphingomyelin, were much higher. Only in the case of the NFA galactocerebroside and at low mole fractions of sphingomyelin was immiscibility detected. Ternary mixtures of the two cerebrosides with sphingomyelin/cholesterol and phosphatidylcholine/cholesterol (PC/Chol) showed different miscibility characteristics. On cooling from 80°C all mixtures formed homogeneous gel states. However, on standing the cerebrosides separated into discrete gel phases in all mixtures but one, that in which HFA galactocerebrosides were mixed with sphingomyelin and cholesterol. The cerebroside in the mixture with the composition closest to that of myelin, HFA/PC/Chol, melted at 38°C. On scanning guinea pig CNS myelin which had been equilibrated at 5°C a transition was detected with T_{max} 33°C. On the basis of comparison with the HFA/PC/Chol mixture we propose that the transition in myelin at this temperature is due to the melting of a galactocerebroside gel phase.

Introduction

We have chosen to study the myelin galactocerebrosides because of their involvement in the disease multiple sclerosis. Multiple sclerosis (MS) is an autoimmune disease in which myelin, the insulating membrane surrounding nerve axons, is destroyed while the axons themselves are spared. Of all the biomembranes myelin contains the highest proportion of lipid (75–80%) and of these lipids galactocerebrosides are both the most numerous and most characteristic. It has been shown that (a) antibodies against cerebrosides are present in sera from patients suffering from MS [1], (b) such antibodies demyelinate cerebellar tissue cultures [2] and (c) experimental allergic encephalomyelitis, a model for MS, can be induced

Abbreviations: NFA and HFA galactocerebrosides, galactocerebrosides with unsubstituted and α-hydroxy-substituted acyl chains, respectively; CNS, central nervous system; MS, multiple sclerosis.

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in rabbits more readily if they are inoculated with galactocerebroside as well as myelin basic protein [3]. Epidemiological studies have shown that some individuals are more susceptible to this disease than others. Susceptibility is under multigenic control [4] and although at this time it is not clear how susceptibility is manifest at the molecular level, there is a body of opinion for a defect in membrane structure [5]. Such a defect could involve the galactocerebrosides.

There are two types of galactocerebroside in brain, those which possess an α-hydroxy substituent on the acyl chain (HFA) and those that do not (NFA). Studies using mice of various ages have shown that the ratio of NFA to HFA is large in immature brain but decreases with age until it is the HFA lipid which predominates in the adult [6,7]. The total amount of cerebroside in brain increases steadily with age until maturity. In the peripheral nervous system the cerebroside composition more closely resembles that of the immature central nervous system, i.e. low total cerebroside, high proportion of NFA [8]. To gain an idea of the role of the galactocerebrosides in determining the physical properties of myelin and the effect on these properties of changes in the quantity and type of cerebroside present, we have studied the thermotropic behaviour of binary and tertiary mixtures of NFA and HFA galactocerebrosides with the other main brain lipids.

Several other authors [8–16] have also published calorimetric studies of cerebroside mixtures but this work has either been confined to synthetic lipids or the range of mixtures studied is small so that at present it is not possible to get an insight into the likely state of cerebroside within myelin.

Materials and Methods

Materials

Galactocerebrosides with unsubstituted (NFA) and α-hydroxy-substituted acyl chains (HFA) were purchased from the Sigma Chemical Co. They had been prepared by chromatographic separation of lipids extracted from bovine brain. The contamination of each cerebroside fraction by the other was claimed to be less than 2% and the total cerebroside content of each fraction was stated to be 99%. An analysis of the acyl chains in each group of lipids has been published [17]. Average molecular weights calculated for NFA and HFA galactocerebrosides were 808 and 801, respectively.

Phosphatidylcholines and sphingomyelins were purchased from Sigma and are bovine brain extracts. Average molecular weights have been calculated for them from published acyl chain analyses and are 762 and 795, respectively [18–21].

Cholesterol, puriss grade (reputedly > 98%), was purchased from Fluka, AG.

The purity of all lipids was checked by thin-layer chromatography on silica gel plates. Various amounts of the lipids were spotted onto the base of the plate and it was eluted with a mixture of chloroform and methanol. The ratio of the two solvents was chosen so that each lipid had an R_f of 0.5. Cerebrosides were visualised by spraying their plates with an orcinol solution. All other chromatograms were sprayed with a 0.1% solution of Rhodamine 6G in ethanol. No lipids were used in this study if anything other than a single spot appeared in their chromatograms.

Lyophilised guinea pig CNS myelin was a gift from Dr. R. King of the Neurological Science Department R.F.H.S.M.

Methods

Lipid mixtures were prepared by dissolving preweighed amounts of the components in chloroform/methanol and removing the solvent by warming to 35°C under a nitrogen atmosphere. The mixtures were then stood under vacuum for 24 h. Samples for calorimetry (5–30 mg) were weighed on a Perkin-Elmer AD4 electronic microbalance into stainless steel high pressure calorimetry pans. The lipids were hydrated with excess water and the pans hermetically sealed. Heating and cooling scans were carried out on a Perkin-Elmer DSC7 scanning calorimeter interfaced to a Perkin-Elmer 7700 computer via a TAC7 instrument controller. Heat flow versus temperature data were digitised and stored on the computers hard disk. The temperature at which the heat flow in a transition was at a maximum (T_{max}) and the enthalpy changes of transitions were calculated using software supplied by the Perkin-Elmer Co. The temperature scale was calibrated using cyclohe-

xane and indium as standards and the enthalpy changes were quantified by comparison with indium (ΔH 28.45 J/g). Unless otherwise stated all samples were scanned at 2.5 C°/min. Samples were repeatedly heated to 100 °C and then cooled to 0 °C until reproducible behaviour was obtained. Whenever no transition was detected in the first heating scan or the enthalpy change on cooling was less than the enthalpy change on heating the sample was equilibrated for up to a week at room temperature or overnight at 5 °C and then rescanned.

Results and Discussion

(a) NFA galactocerebrosides and cholesterol

When a sample of NFA galactocerebroside which had been equilibrated for 24 h was heated it melted in a highly cooperative manner with a large change in enthalpy content, 90.3 J/g (see Fig. 1 and Table I). However, re-formation of the gel state on cooling took place in a far less cooperative manner. There were two broad exotherms in the cooling curve and the sum of the enthalpy changes of these transitions, 32.8 J/g, was much less than the enthalpy change observed on melting. Clearly the melting process was not rapidly reversible and a metastable gel state of higher energy content than the initial gel state had been formed. Heating the cerebrosides in this state produced a more complex heating scan than that observed initially. There was an exotherm at low temperature, indicating partial relaxation of the high energy metastable gel state and the main melting endotherm was split into two components. The second cooling curve was identical to the first. If the sample was equilibrated for 24 h and scanned again, a heating curve with a single cooperative endotherm, similar to that found initially, was obtained. The formation of metastable gel states by NFA galacto- and glucocerebrosides has been reported by several groups and the results obtained here agree well with these previous reports [22–24].

Addition of cholesterol to NFA galactocerebroside bilayers produced marked changes in their thermal behaviour. In general multiple endotherms appeared in the heating scans, the number of cooling exotherms was decreased and

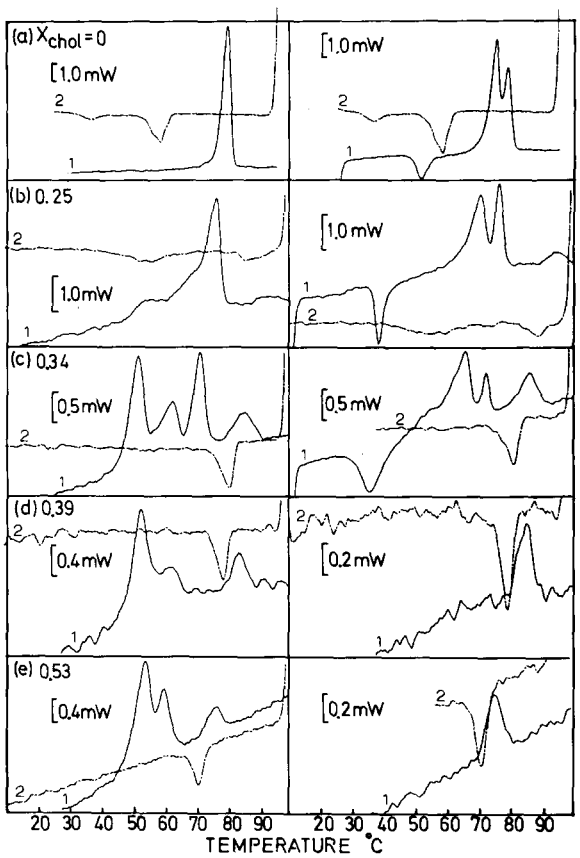

Fig. 1. Thermograms of mixtures of NFA galactocerebrosides with cholesterol. The solid traces (1) are heating scans and the broken traces (2), cooling scans. Measurement of the thermograms on the right hand side of the figure were commenced immediately the cooling scans shown on the left had ended. The mole fractions of cholesterol in each mixture are shown on each set of thermograms.

there was a reduction in the enthalpy change of all transitions.

At a cholesterol mole fraction of 0.25 the T_{max} values of the main endotherms in the heating scans lay 3–4 C° below the T_{max} values of the pure lipid and enthalpy changes were reduced by 28% in the case of the initial scan and 48% in the reheat. In addition a new transition at very high temperature appeared (at 94 °C) which was more pronounced in the reheating scan. Unlike the other transitions it was completely reversible. At 0.25 mole fraction the main cooling exotherm was very broad and its enthalpy change reduced by 70% compared to the same transition in the pure lipid.

At 0.34 mole fraction cholesterol there was a

TABLE I

T_{max} VALUES (°C) AND ENTHALPY CHANGES (J/g) FOR PHASE TRANSITIONS OF NFA GALACTOCEREBROSIDE/CHOLESTEROL MIXTURES

X_{Chol}	1st scan				2nd scan			
	heat		cool		heat		cool	
	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH
0.0	78.9	+90.3	58.4	−26.2	51.8	−12.0	58.3	−26.8
		+90.3 [a]	36.2	−7.6	74.8, 78.7	+85.0	36.2	−7.8
0.25	76.0	+61.7	86.0	−2.9	38.5	−11.8	88.5	−3.5
	93.6	+3.4	53.0	−10.3	70.2, 76.1	+44.3	54.3	−11.4
		+65.1 [a]			93.8	+4.1		
0.34	52.6, 63.5, 72.0	+47.1	80.9	−7.2	35.6	−11.0	80.9	−7.3
	86.0	+6.6			65.7, 72.2	+20.8		
		+53.7 [a]			86.0	+6.5		
0.39	53.4, 63.5	+31.4	79.3	−7.9	85.1	+8.2	79.6	−10.0
	84.5	+7.4						
		+38.8 [a]						
0.53	53.9, 60.4	+40.8	71.0	−6.8	75.0	+7.0	71.2	−6.4
	76.4	+5.3						
		+46.1 [a]						

[a] Denotes sum of enthalpy changes at this mole fraction of cholesterol.

further reduction in T_{max} and ΔH of the main melting transition in both first and second heating scans. In addition the main endotherm in the first heating scan was split into three distinct peaks. The high-temperature transition detected at 0.25 mole fraction cholesterol shifted to a slightly lower temperature and its enthalpy change was doubled. In the cooling scans the only exotherm detectable (at 86 °C) was that associated with the high-temperature transition.

Increasing the cholesterol content to 0.39 mole fraction removed the peak at 72 °C in the first heating scan and all transitions from the second heating scan except the reversible transition which appeared at 0.25 mol fraction cholesterol. Further increases in cholesterol content had no effect on the general form of the thermograms until 0.75 mole fraction cholesterol when a broad transition appeared around 80 °C (data not shown) indicating the presence of a pure cholesterol phase.

After completion of the second cooling scan the mixtures were equilibrated for 24 h at room temperature. The heating and cooling scans measured after equilibration were identical to the original scans shown on the left hand side of figure one.

Shipley and Ruocco [13] have studied the thermal properties of mixtures of *N*-palmitoylgalactocerebroside and cholesterol by differential scanning calorimetry and X-ray diffraction. They concluded that cerebroside and cholesterol were immisible in the gel state and that when heated melting of the cerebroside commenced at the phase boundaries thereby reducing its melting temperature by 20–30 °C. In the liquid crystalline state cerebroside and cholesterol were found to be miscible.

Such an hypothesis would also partly explain our observations of the thermotropic behaviour of the mixture of natural NFA galactocerebrosides and cholesterol. Examining the results for the 0.39 mole fraction cholesterol mixture and for the moment ignoring the reversible transition, it can be seen that the bulk of the cerebroside in this mixture melts well below the melting temperature of the pure lipid. Similarly to Shipley and Ruocco we conclude that NFA-galactocerebrosides and cholesterol are immiscible in the gel state and the endotherm with peaks at 53.4° C and 63.5° C (Fig. 1) corresponds to the melting of the cerebroside to form a liquid crystalline state in which cerebroside and cholesterol are mixed homogeneously. The absence of a corresponding exotherm in the cooling curves demonstrates that cerebroside and cholesterol remained mixed when cooled at the scanning rate employed in these experiments since it has been shown that when cholesterol is miscible with a lipid which has a gel-liquid crystal phase transition, it reduces the cooperativity and ΔH of that transition [25]. There was no endotherm in a heating scan run immediately after the initial cooling scan. An endotherm identical to the one originally found did reappear if the sample was stood at room temperature for 24 h. Evidently during this time slow phase separation of cerebroside and cholesterol took place in the gel state.

Returning to the high temperature reversible transition; the exotherm at 79.3° C in the cooling scans of this mixture suggests that intermolecular forces are sufficiently strong between some of the cerebroside species for their phase separation from cholesterol on solidification not to be rate limited.

0.39 was the lowest cholesterol mole fraction at which the melting behaviour of pure cerebroside was no longer evident. At 0.34 mole fraction the endotherm with T_{max} 72° C in the initial heating scan and the exotherm (35.6° C) and the twin-peaked endotherm (65.7, 72.2° C) in the reheating scan are characteristic of the thermotropic behaviour of the pure cerebroside.

(b) HFA galactocerebroside and cholesterol

HFA galactocerebrosides did not form a metastable gel state in the manner of the NFA galactocerebrosides. However, on cooling not all the hydroxy-cerebroside returned immediately to the original gel state. In the initial thermogram the ΔH of the melting endotherm was 59.9 J/g while the ΔH of the solidification exotherm was only 31.6 J/g (Table II). As Fig. 2 shows the initial melting endotherm of the cerebroside was two-peaked and it was the part of the sample which gave rise to the low-temperature peak which did not immediately return to the original solid state. In the reheating scan a single-peaked endotherm was found, the T_{max} of which corresponded exactly with the peak in the initial scan which lay at higher temperature.

Addition of cholesterol shifted the melting endotherm of the cerebroside to lower temperature and reduced the enthalpy change of the transition.

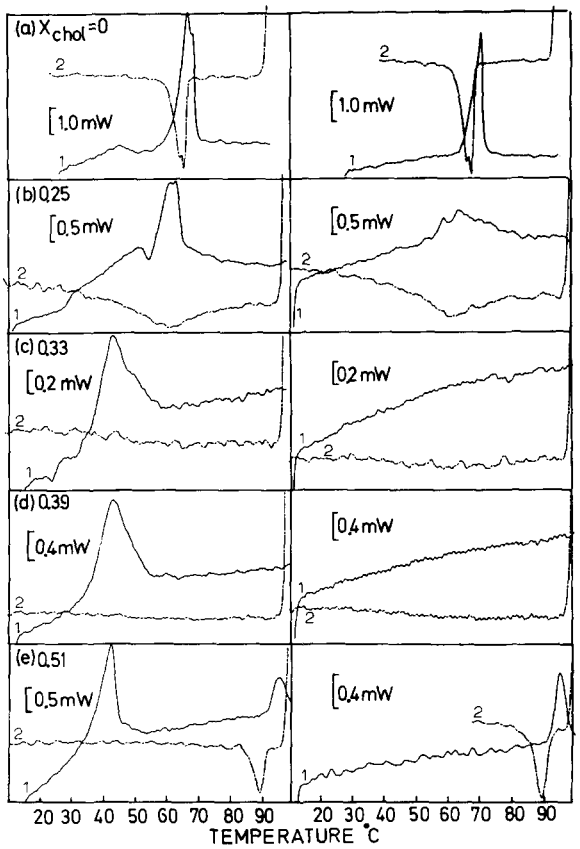

Fig. 2. Thermograms of mixtures of HFA galactocerebrosides with cholesterol. The solid traces (1) are heating scans and the broken traces (2), cooling scans. Measurement of the thermograms on the right hand side of the figure were commenced immediately the cooling scans shown on the left had ended. The mole fractions of cholesterol in each mixture are shown on each set of thermograms.

TABLE II

T_{max} VALUES (°C) AND ENTHALPY CHANGES (J/g) FOR PHASE TRANSITIONS OF HFA GALACTOCEREBROSIDE/CHOLESTEROL MIXTURES

X_{Chol}	1st scan				2nd scan			
	heat		cool		heat		cool	
	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH
0.0	68.9	+59.9	67.9	−31.6	70.5	+31.8	67.9	−30.0
0.25	52.9 \ 63.2 /	+31.5	62.0	−16.3	63.6	+11.3	60.5	−10.7
0.33	44.2	+29.0	–	–	–	–	–	–
0.39	44.0	+26.1	–	–	–	–	–	–
0.51	42.5	+20.0	90.1	−6.3	94.4	+5.5	89.2	−7.4

At 0.33 mole fraction cholesterol the cerebroside phase melted at 44.2°C, 24.7°C below T_{max} of the pure lipid. Further addition of cholesterol up to a mole fraction of 0.77 (data not shown) had little effect on the T_{max} and relatively little effect on the ΔH of this transition. At 0.77 mole fraction cholesterol transitions characteristic of a pure cholesterol phase were seen in the heating and cooling scans. At 0.33 and 0.39 mole fractions there was no transition in the cooling scan and the melting endotherm observed initially was not seen when the sample was reheated. If however the sample was stood at 5°C for 24 h before reheating, an endotherm identical to that found initially was observed. In these respects the behaviour of the HFA galactocerebrosides resembled the NFA galactocerebrosides and we believe that both can be explained in the same way: that cerebroside and cholesterol are miscible in the liquid crystalline state but are immiscible in the gel state; however, on cooling from the liquid crystalline state a non-equilibrium homogeneous gel state is formed in which phase separation of cerebroside and cholesterol takes place slowly.

While the interactions of cholesterol with the two cerebrosides were broadly similar there were differences in the cholesterol mole ratios at which the melting behaviour characteristic of the unmixed lipid completely disappeared and the endotherm and exotherm of the high-temperature fully reversible transition appeared. The transition with T_{max} at 72°C in the thermogram of NFA galactocerebrosides at 0.34 mole fraction cholesterol we assign to a melting process similar to that found in the pure lipid. No such transition was found in the thermogram of the HFA mixture of similar composition, simply one transition which persisted at high cholesterol mole fractions and was clearly dependent on the presence of cholesterol. While the high temperature reversible transition was apparent at the very lowest cholesterol mole fraction in the NFA mixture it was not apparent at mole fractions below 0.5 in the HFA mixtures.

The melting temperature and enthalpy change of the HFA cerebrosides were significantly less than the NFA cerebrosides demonstrating that intermolecular forces are weaker between the hydroxy-substituted cerebrosides. This weaker intermolecular interaction was most likely responsible for the disappearance of the pure lipid melting endotherm at the lowest cholesterol mole ratio studied in the HFA galactocerebroside series.

(c) Galactocerebroside / sphingomyelin mixtures

Only one major transition was found in the heating and cooling scans of the mixtures of both cerebrosides with sphingomyelin (see Fig. 3). The T_{max} values of the transitions decreased progressively and approached the values measured for sphingomyelin as the sphingomyelin mole fraction in the mixture was increased (see Table III). This behaviour suggests that sphingomyelin and the cerebrosides are completely miscible in both the gel and liquid crystalline states.

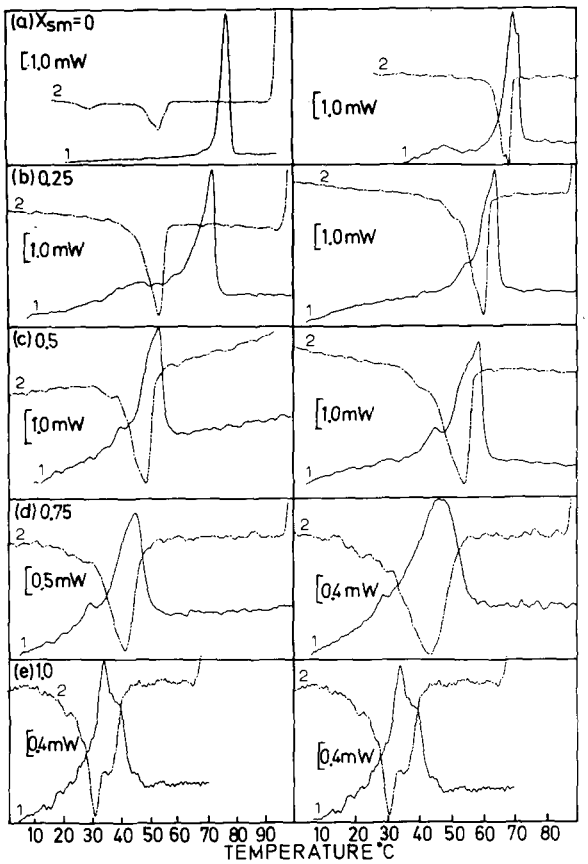

Fig. 3. Thermograms of mixtures of bovine brain sphingomyelin with on the left hand side of the figure, NFA galactocerebrosides and on the right hand side, HFA galactocerebrosides. The solid traces (1) are heating scans and the broken traces (2), cooling scans. The mole fractions of sphingomyelin in each mixture are shown on each set of thermograms.

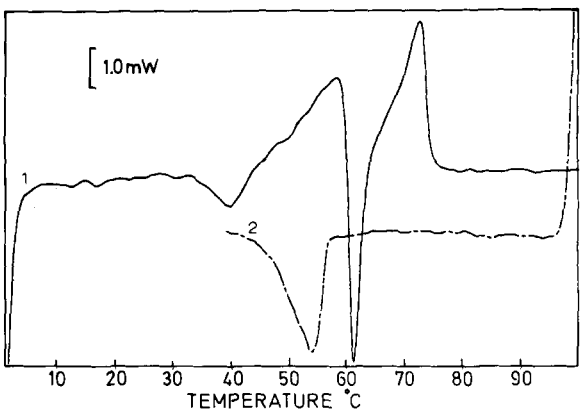

Fig. 4. Thermogram of a mixture of NFA galactocerebrosides and bovine brain sphingomyelins. The mole fraction of sphingomyelin was 0.25. The solid trace (1) is the heating scan and the broken trace (2), the cooling scan. The mixture was heated to 80 °C before scanning commenced. The T_{max} values and enthalpy changes for the exotherms are, heating 39.2 °C, −9.3 J/g and cooling, 53.6 °C, −32.2 J/g, respectively.

Only in the case of the NFA galactocerebroside mixture containing 0.25 mole fraction sphingomyelin was there evidence of immiscibility. In all other mixtures the ΔH values of the transitions seen in the heating and cooling scans were equal. However in this mixture the enthalpy change during the initial cooling scan was approximately half that observed on heating. The gel state formed on cooling was not the equilibrium state. The scan obtained on heating this state (Fig. 4) was very different from the initial scan. There was an exotherm at 39.2 °C, an endotherm, another highly

TABLE III

T_{max} VALUES (°C) AND ENTHALPY CHANGES (J/g) FOR PHASE TRANSITIONS OF GALACTOCEREBROSIDE/SPHINGOMYELIN MIXTURES

X_{Sm}	NFA galactocerebroside				HFA galactocerebroside			
	heat		cool		heat		cool	
	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH
0.0	78.9	+90.3	58.4	−26.2	68.9	+59.9	67.9	−31.6
			36.2	−7.6				
0.25	71.7		53.5	−33.6	63.6	+39.0	60.1	−35.7
	44.6	+63.0						
0.50	52.9	+35.7	48.6	−35.5	58.2	+36.0	54.1	−35.1
0.75	44.3	+32.9	41.1	−38.4	45.9	+36.0	44.0	−34.4
1.0	33.8	+34.9	30.6	−34.2				

cooperative exotherm (T_{max} 58.8°C) and finally another endotherm. The following cooling scan was identical to the one obtained initially. We conclude that the liquid crystalline state of this mixture is homogeneous and that some of the lipids remain mixed after they enter the gel state on cooling, the enthalpy change and/or cooperativity of the transition on formation of a mixed gel state being less than formation of a gel by either pure lipid. However because of the strong intermolecular forces between molecules of NFA galactocerebrosides, at this relatively low sphingomyelin mole ratio we believe the equilibrium state is the one in which there are separate phases and that the sharp exotherm in the second heating scan is caused by phase separation and solidification of the cerebroside. The initial heating scan obtained by heating the equilibrium gel state (see Fig. 3) had a small endotherm with a T_{max} at 44.6°C. In accordance with the hypothesis above we assign this endotherm to the melting of a phase which is predominantly sphingomyelin.

(d) Galactocerebroside / phosphatidylcholine mixtures

Although both possess the phosphorylcholine polar group, the thermal properties of bovine brain sphingomyelins and phosphatidylcholines were very different. While the sphingomyelins melted around 34°C, the melting temperature of the phosphatidylcholines was so low we were unable to measure it accurately. Carrying out experiments in water/ethylene glycol (1 : 1, v/v) showed that T_{max} for bovine brain phosphatidylcholines was below −30°C. This is presumably a consequence of the high oleoyl acyl chain content of bovine brain phosphatidylcholines [20] (ethanolamine phosphatides from bovine brain contain an even higher proportion of unsaturated (54%), and polyunsaturated acyl chains (32.7%)). As well as the differences in properties of the pure lipids, their mixtures with the cerebrosides also displayed characteristically different thermal properties. As the proportion of the phosphatidylcholine in mixtures with both cerebrosides was increased, T_{max} of the heating endotherm shifted to lower temperature and ΔH fell (Fig. 5 and Table IV). However, unlike the sphingomyelin mixtures T_{max} did not approach the melting temperature of the phosphatidylcholines at high phosphatidylcholine content and the cooperativity of the transitions fell progressively, especially in the case of HFA, as the phosphatidylcholine content increased. This behaviour suggests that the cerebroside and phosphatidylcholine, unlike sphingomyelin, are at most only partially miscible in the gel state. Ruocco et al. [12] who studied dipalmitoylphosphatidylcholine and Curatolo [16] who studied 1-palmitoyl-2-oleoylphosphatidylcholine also came to the conclusion that the solubility of phosphatidylcholine in cerebroside is limited. Clearly there is a stronger interaction between sphingomyelin and cerebroside than between phosphatidylcholine and cerebroside.

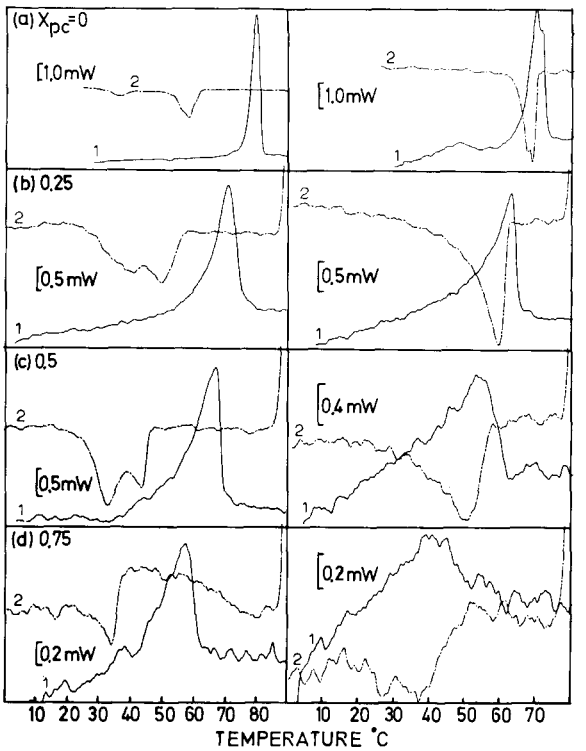

Fig. 5. Thermograms of mixtures of bovine brain phosphatidylcholines with on the left hand side of the figure, NFA galactocerebrosides and on the right hand side, HFA galactocerebrosides. The solid traces (1) are heating scans and the broken traces (2), cooling scans. The mole fractions of phosphatidylcholine in each mixture are shown on the thermograms.

As figure five shows and as discussed earlier when pure NFA galactocerebrosides were cooled the cooling scan contained two exotherms. The

TABLE IV

T_{max} VALUES (°C) AND ENTHALPY CHANGES (J/g) FOR PHASE TRANSITIONS OF GALACTOCEREBROSIDE/PHOSPHATIDYLCHOLINE MIXTURES

X_{PC}	NFA galactocerebroside				HFA galactocerebroside			
	heat		cool		heat		cool	
	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH	T_{max}	ΔH
0.0	78.9	+90.3	58.4	−26.2	68.9	+59.9	67.9	−31.6
			36.2	−7.6				
0.25	70.8	+49.5	50.1 \		61.9	+33.1	58.8	−32.3
			41.3 /	−43.8				
0.5	67.0	+35.2	44.4 \		51.9	+18.2	49.9	−18.3
			33.4 /	−22.4				
0.75	57.5	+10.9	34.4	−4.2	40.2	+7.8	37.0	−9.5

exotherm at the highest temperature (T_{max} 58.4° C) had two components. There was no low-temperature transition in the cooling scan of the 0.25 mole fraction phosphatidylcholine mixture. However, the relative size of the low-temperature component in the transition that remained was increased. It was further increased in the 0.5 mole fraction mixture. However, at 0.75 mole fraction this component was absent and the enthalpy change on cooling was less than half that measured on heating. A metastable high-energy gel state was formed. As with the other metastable gel states formed by

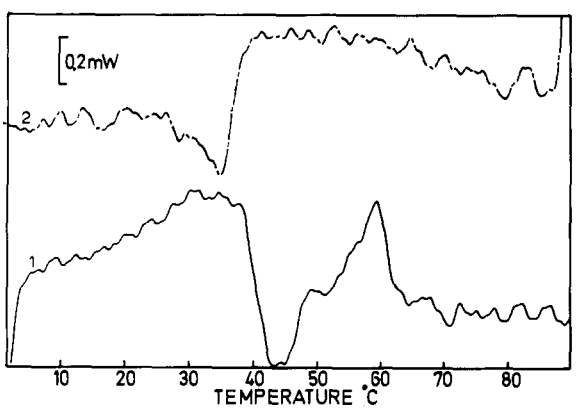

Fig. 6. Thermogram of a mixture of NFA galactocerebrosides and bovine brain phosphatidylcholines. The mole fraction of phosphatidylcholines was 0.75. The solid line (1) is the heating scan and the broken line (2), the cooling scan. The mixture was heated to 80° C before scanning commenced. The T_{max} and enthalpy change of the cooling exotherm are 34.6° C and −4.4 J/g, respectively.

NFA galactocerebrosides, on heating relaxation took place and in the second heating scan there was an exotherm (T_{max} 42.9° C) (see Fig. 6). The stable gel state was also reformed if the sample was allowed to stand at room temperature for 24 h. We conclude that as with cholesterol, NFA galactocerebrosides and the phosphatidylcholines are miscible in the liquid crystalline state but at equilibrium virtually immiscible in the gel state. However, at high phosphatidylcholine contents a fraction of the cerebroside appears to enter the gel state still mixed with phosphatidylcholine in a transition which is either highly uncooperative or has a low ΔH. On heating phase separation and solidification of the cerebroside occurs producing an exotherm in the reheat scan.

(e) Ternary mixtures of the cerebrosides with phosphatidylcholine, sphingomyelin and cholesterol

The thermal characteristics of 1 mole NFA galactocerebrosides mixed with (1) 1 mole of sphingomyelin and 2 moles of cholesterol and (b) 1 mole phosphatidylcholine and 2 moles of cholesterol are shown in Fig. 7. The heating scans of both mixtures possessed endotherms at approximately the same temperature (58° C) but the enthalpy change in the phosphatidylcholine mixture was twice that found for the sphingomyelin mixture. The results demonstrate that in the gel state of these mixtures a significant proportion of the cerebroside exists in a separate phase or a phase only diluted to a minor degree with the

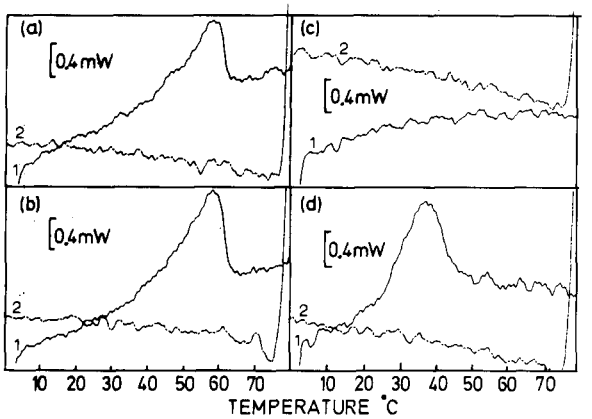

Fig. 7. Thermograms of the ternary mixtures (mole ratio 1 : 1 : 2): (a) NFA galactocerebrosides/sphingomyelins/cholesterol; (b) NFA galactocerebrosides/phosphatidylcholines/cholesterol; (c) HFA galactocerebrosides/sphingomyelins/cholesterol; and (d) HFA galactocerebrosides/phosphatidylcholines/cholesterol. The solid traces (1) are heating scans and the broken traces, (2), cooling scans. The T_{max} values and enthalpy changes for the endotherms are: (a) 57.6 °C, 8.6 J/g (b) 57.8 °C, 16.2 J/g (d) 37.2 °C, 12.5 J/g.

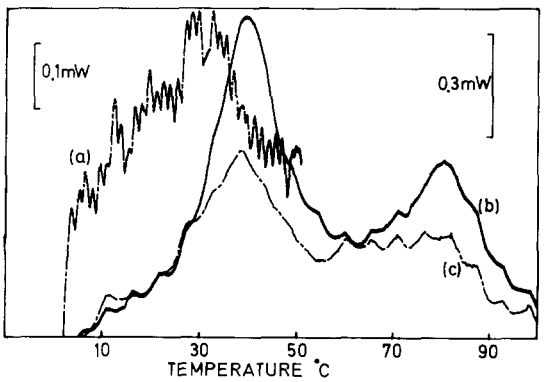

Fig. 8. Thermogram of the guinea pig central nervous system myelin. Heating scans. (a) 2.5 C°/min: T_{max} 32.7 °C, ΔH 1.1 J/g. (b) 10 C°/min. (c) 10 °C/min: heated to 100 °C and then allowed to stand for two weeks at 5 °C.

other constituents of the mixture. The greater enthalpy change of the phosphatidylcholine mixture is most likely a reflection of the lower solubility of this lipid in the cerebroside phase.

The behaviour of HFA galactocerebroside mixtures of similar composition is shown in (c) and (d) of Fig. 7. There was a striking difference between the thermograms. The heating scan of the spingomyelin mixture did not contain an endotherm demonstrating that in the equilibrium gel state the lipids in this mixture were homogeneously mixed and there was no separate cerebroside phase. In this mixture the weaker intermolecular forces of the hydroxycerebroside appear to be completely counterbalanced by interactions with the other lipids. Interactions between the hydroxycerebroside and phosphatidylcholine are not as strong as the interaction with sphingomyelin and in mixture (d) a separate cerebroside phase was present in the gel state at equilibrium.

The mixtures which have a separate cerebroside phase in the gel state did not return to this state immediately when they were cooled as there was no evidence of exotherms in the cooling scans. We believe that the same mechanism is responsible for hysteresis in the transitions of the ternary mixtures as the binary: that a homogeneous liquid crystalline state does not rapidly reform the equilibrium heterogeneous gel state on cooling. If the ternary mixtures (a), (b) and (c) were allowed to stand for a week before reheating, endotherms similar to those shown in Fig. 7 appeared in the heating scans. The time to return to equilibrium could be reduced to 24 h if the mixtures were stood at 5 °C rather than room temperature.

(f) Central nervous system myelin

Scan (a) shown in Fig. 8 was made on guinea pig central nervous system myelin which had been equilibrated at 5 °C for 8 weeks. Although the signal to noise ratio was low there was clearly a reversal in the direction of change of heat capacity and a transition around 33 °C. Moscarello et al. [26] used a microcalorimeter to study human CNS myelin and have published thermograms which have a higher signal to noise ratio than thermogram (a). They also detected a transition at 33 °C.

To improve the signal to noise ratio we increased the scanning speed to 10 C°/min and obtained thermogram (b) in Fig. 8. The transition observed at the slower scanning rate was again seen, this time more clearly resolved. However, at the higher scanning speed T_{max} was shifted from 33 to 39 °C. Scanning was continued to 100 °C and a second transition was detected at 80 °C. Moscarello et al. [26] also detected a second transition near this temperature. An exotherm indicative of a cooperative solidification was not observed when the sample was cooled from 100 °C,

and the two endotherms seen in trace (b) were not seen in a heating scan made at the conclusion of the first heating-cooling cycle. However, the endotherms were observed when the sample was equilibrated at 5 °C before rescanning. Thermogram (c) was obtained with a sample equilibrated for two weeks after an initial heating to 100 °C.

There are a number of similarities between the thermotropic behaviour of myelin and the ternary cerebroside mixtures. The mixture with the composition closest to myelin, HFA galactocerebroside/phosphatidylcholine/cholesterol had a melting endotherm with T_{max} 37.2 °C, near the T_{max} of the low-temperature endotherm of myelin. Like myelin no cooperative solidification was detected when the mixture was cooled and an endotherm was not found when the mixture was immediately reheated. However an endotherm was observed if the mixture was equilibrated at 5 °C before reheating. These similarities suggest that the low temperature endotherm in the myelin thermogram is the melting of a crystalline cerebroside gel phase. At body temperature, 37 °C, the galactocerebrosides are homogeneously mixed with the other myelin membrane components, but evidently when the membrane was held at 5 °C cerebrosides separated into a crystalline gel phase. The smallest and largest enthalpy changes observed on the melting of cerebroside in the ternary mixtures were 8.6 J/g and 16.2 J/g. It would be expected that if the cerebroside in myelin did separate into a crystalline gel state there would be a similar enthalpy change on the melting of this state. Since galactocerebrosides make up approx. 16% of the weight of myelin this would imply an enthalpy change of between 0.5 and 0.9 J/g of myelin. The value for the low temperature transition in guinea pig CNS myelin is 1.1 J/g, close to the range of values expected and further evidence to support our contention that the origin of this transition is the melting of the myelin cerebrosides.

Conclusion

The results presented here show that in the gel state cerebrosides are relatively immiscible with the other brain lipids. Miscibility is at its greatest with the other main sphingolipid, sphingomyelin, but even here at low sphingomyelin mole fractions, sphingomyelin and the NFA galactocerebrosides exist as two phases at equilibrium in the gel state. However, cerebrosides appear to be miscible with all the lipids examined in the liquid crystalline state for they show a tendency to form metastable homogeneous gel states when cooled rapidly from temperatures above their melting point. Phase separation is often a slow process on the time scale of the experiments carried out here.

The reason for immiscibility is undoubtedly the very strong attractive forces between cerebroside molecules in the gel state as evidenced by their high melting temperatures. The hydroxy-substituted cerebrosides melt at a temperature 10 C° below that of the unsubstituted species and this temperature, 68 °C, although high when compared with the other brain lipids, suggests that intermolecular forces between hydroxycerebrosides are not quite as strong as those which exist in the unsubstituted fraction. This conclusion is borne out by the differences found in the thermotropic behaviour of the mixtures examined here. HFA galactocerebroside mixtures melt at lower temperatures than their NFA counterparts and HFA galactocerebrosides are miscible with sphingomyelin in all proportions and with sphingomyelin/cholesterol mixtures while NFA galactocerebrosides are not. Our work therefore suggests a reason for the high HFA/NFA galactocerebroside ratio found in membranes which contain relatively high proportions of cerebroside. A high HFA content will reduce the propensity of the cerebroside to form a separate gel phase. The formation of such a phase would be expected to reduce the stability of the membrane. In CNS myelin the ratio of NFA to HFA galactocerebrosides and the total cerebroside in the membrane are the maximum possible, for any further increase in either of these quantities would lead to the separation of a discrete cerebroside gel phase at 37 °C. This high concentration of cerebroside is in keeping with the role of the myelin membrane as an insulator since it has been shown that the incorporation of cerebroside into phosphatidylcholine bilayers increases their electrical resistance [9].

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