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Differential scanning calorimetric and Fourier transform infrared spectroscopic investigations of cerebroside polymorphism

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Calorimetric and Fourier transform infrared (FTIR) spectroscopic studies have been made of the polymorphism exhibited by bovine brain cerebroside–water systems, and the effect of cholesterol and dipalmitoylphosphatidylcholine (DPPC) upon this polymorphism was investigated. The conversion of the cerebroside from the thermodynamically stable to the metastable form is found to be accompanied by spectral changes, indicating a decrease in cerebroside headgroup hydration and a rearrangement of the hydrogen-bond network. The incorporation of low concentrations of cholesterol and DPPC into cerebroside bilayers broadens the thermal transitions associated with the cerebroside as a result of the disruption of cerebroside–cerebroside interactions. This disruption is evident in the spectra of cerebroside/cholesterol mixtures.

Introduction

Cerebrosides belong to a class of lipid known as glycosphingolipids. These lipids, composed of a sphingosine backbone, a fatty acid (either hydroxy-substituted or unsubstituted), and one or more hexose sugar residues, are found in most, if not all, animal cell membranes, albeit in relatively small amounts. Appreciable amounts are found in certain severe pathological conditions (Gaucher's disease, Krabbe's disease, metachromic leucodystrophy) and in certain specialised tissues under normal circumstances. A good example of the latter is the myelin sheath, which contains high level of cerebrosides. The function of cerebrosides in such tissues is not clear, but it is likely that they are involved in maintaining membrane integrity.

Cerebrosides and many other glycosphingolipids exhibit complex polymorphic phase behaviour. The structure and polymorphism of non-hydroxy fatty acid galactocerebrosides (type II galactocerebrosides) have been the subject of a number of studies in recent years, using such techniques as DSC [1,2], X-ray diffraction [3] and spectroscopy [4–6]. At present, the thermotropic mesomorphism is believed to be due to rearrangement of hydrogen-bond networks [3] and changes in hydration [3,7].

Upon heating type II galactocerebrosides in excess water, the main gel-liquid crystalline phase transition occurs at around 80 °C. Rapid cooling of this phase produces a gel state with an X-ray

Abbreviations: FTIRS, Fourier transform infrared; DPPC, 1,2-dipalmitoyl-1-*sn*-glycero-3-phosphorylcholine; DSC, differential scanning calorimetry.

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diffraction pattern identical to that of an anhydrous sample [3]. Subsequent heating of this phase results in an exothermic transition over the range 50–60 °C followed by the main endotherm at 80 °C. The exothermic nature of the first transition indicates conversion from a high-energy metastable state to a lower-energy stable state.

As cerebrosides are the major polar lipid in central and peripheral nervous system myelin, it is apparent that a more complete understanding of this polymorphism and how other myelin components affect it is desirable.

Materials and Methods

Type II bovine brain cerebrosides (98% non-hydroxy fatty acids, 99% pure) and cholesterol (5(6)-cholesten-3β-ol, 99% pure) were purchased from Sigma. 1.2-Dipalmitoyl-1-*sn*-glycero-3-phosphorylcholine (DPPC, 99% pure) was purchased from Fluka. All solvents were of spectrophotometric grade.

DSC thermograms were recorded on a Perkin-Elmer DSC7 at heating and cooling rates of 2.5 C°/min over the range 0–100 °C. Lipid mixtures were prepared as below and anhydrous material weighed into a stainless steel pan. Each sample (15–20 mg) was then hydrated by introducing excess water via a microsyringe and the pans were then sealed. To ensure that the samples were homogeneous and at equilibrium they were taken through several heating and cooling cycles and left to stand for 24 h before being scanned.

IR spectra were recorded on a Perkin-Elmer 1750 FTIR spectrometer continuously purged with dry air at a flow rate of 100 l/min. Samples were mounted in a Beckman FH-01 CFT cell fitted with CaF_2 windows separated by a 6 μm tin spacer. Temperature was maintained at 20 °C using a circulating water jacket. For each sample, 200 scans were recorded at 4 cm^{-1} resolution and were signal averaged. Difference spectra were generated by digital subtraction of a suitable background spectrum on a Perkin-Elmer computer.

Samples were prepared for FTIR spectroscopy as follows. Stock solutions of galactocerebroside, cholesterol and DPPC were made up in chloroform/methanol (2 : 1). Cerebroside/cholesterol and cerebroside/DPPC dispersions were formed by mixing the appropriate volumes of each solution to produce the ratios required. The solvent was then evaporated under N_2 and the samples stored under vacuum to remove any final traces of solvent.

Hydration of samples was achieved by heating lipid and water (cerebroside concentration = 10 mg/ml) at 90 °C and agitating with a vortex mixer. Metastable samples were produced by quenching hydrated mixtures from 90 to 0 °C in an ice bath. Stable samples were produced by incubation of metastable samples at 70 °C for 10 min.

Spectra of anhydrous galactocerebrosides were obtained from a KBr disc containing 1% galactocerebrosides by weight.

Results

Differential scanning calorimetry

Calorimetric thermograms of hydrated galactocerebrosides and galactocerebroside/cholesterol mixtures are presented in Fig. 1. Upon heating the pure cerebrosides, the main gel-liquid crystalline phase transition occurs at 79 °C, with an enthalpy change of 90 J/g. Cooling scans show two exothermal transitions, at 58.4 °C and 36.2 °C with enthalpy changes of −26.2 J/g and −7.61 J/g, respectively, indicating formation of two gel states. The disparity in enthalpy changes of the heating and cooling scans demonstrates that at least one of the gel forms is metastable. A further heating scan (Fig. 1c) shows a distinct exotherm centered at 51.8 °C which has an enthalpy change of −12 J/g. This is followed by a split endotherm, with peaks at 74.8 and 78.7 °C and a combined enthalpy change of 85 J/g. A second cooling scan was identical to the first.

Addition of 9 mol% cholesterol has little effect upon the first heating scan but produces a complex exotherm in the cooling scan with an enthalpy change of −40 J/g and a peak at 57.3 °C (Fig. 1e). A subsequent heating scan (Fig. 1f) shows the characteristic exotherm, reduced in peak temperature to 46.6 °C but of comparable enthalpy to the pure cerebroside. Again, a split endotherm is seen, with peaks at 73.6 °C and 79 °C and an enthalpy change of 70 J/g. This reduction in enthalpy change upon addition of

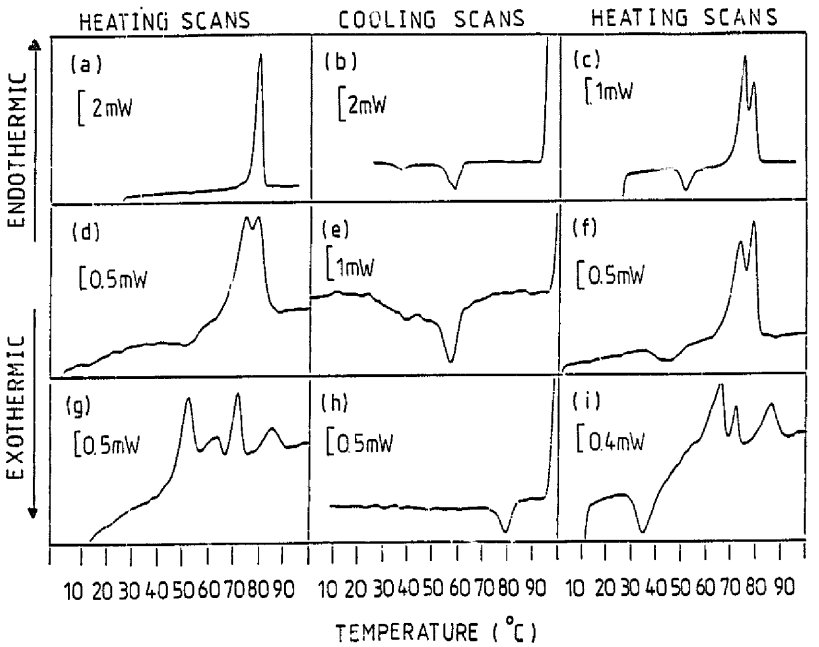

Fig. 1. DSC thermograms of hydrated type II galactocerebrosides containing (a–c) 0; (d–f) 9; and (g–i) 34 mol% cholesterol. Heating and cooling rate = 2.5 C°/min. See text for equilibration protocol.

cholesterol is similar to that reported elsewhere [8].

Thermograms of cerebrosides with the addition of 34 mol% cholesterol are shown in Figs. 1g–i. An initial heating scan exhibits four endothermic transitions centered at 53.2, 63.5, 72 and 86°C with combined enthalpy changes of 53.3 J/g. A cooling scan showed only one exotherm centered at 80.8°C with an enthalpy change of −7.3 J/g. Immediate reheating produced an exotherm at 35.6°C followed by three endotherms at 65.7, 72.2 and 86°C. Heating 24 h after cooling produces a thermogram similar to that measured initially.

The effects of addition of various amounts of DPPC to hydrated galactocerebrosides is shown in Fig. 2. After addition of 9 mol% DPPC, an initial heating scan exhibits an endotherm at 79.3°C with an enthalpy change of 76.2 J/g. Cooling produces an exotherm with a peak at 57.5°C and a shoulder at 55°C, with an enthalpy change of −32.2 J/g. This enthalpy change is markedly lower than the enthalpy change in the preceding heating scan, suggesting formation of a metastable state. A subsequent heating scan (Fig. 2c) confirmed this; an exotherm centered at 54.5°C with an enthalpy change of −18.6 J/g is observed. This is followed by an endotherm at 75.3°C and a small endothermic peak at 78.9°C with a combined enthalpy change of 77.6 J/g. A cooling scan produced behaviour identical to that in the initial cooling scan. The reduction in enthalpy change of the cerebroside transitions is as reported previously [9].

Increasing the DPPC concentration to 33 mol% produces a more complex heating thermogram (Fig. 2e). Three endothermic peaks are observed at 43.5, 54.5 and 72.3°C with a combined enthalpy change of 51.7 J/g. A cooling curve contains only one exotherm at 52.2°C with an enthalpy change of −41.9 J/g. Immediate reheating produced a thermogram exhibiting endothermic and exothermic transitions. Due to overlapping of these transitions it is not possible to measure enthalpy changes, or to establish precisely the peak temper-

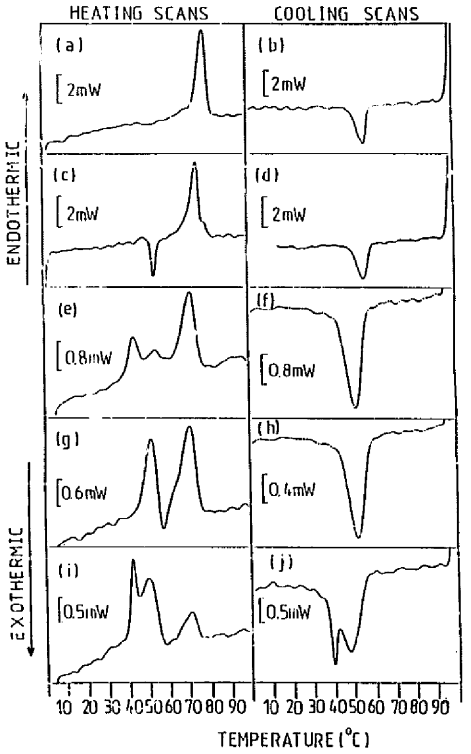

Fig. 2. DSC thermograms of hydrated type II galactocerebrosides containing (a–d) 9; (e–h) 33 and (i–j) 50 mol% DPPC. Heating and cooling rate = 2.5 C°/min. See text for equilibration protocol.

atures of the first two transitions. The final endotherm is observed to have a peak temperature of 71.6°C.

Further elevation of the concentration of DPPC to 50 mol% produces the thermograms shown in Fig. 2i–j. Multiple peaks are seen in heating scans, centred at 42.9, 51.4 and 70.9°C, the combined enthalpy change of the three endotherms being 48.6 J/g. A cooling scan produces a complex thermogram consisting of a broad exotherm at 47.7°C with a shoulder at 52°C and a sharp peak at 39.6°C, the combined enthalpy change being −43 J/g. The observation of three transitions in the cooling scans suggests that phase separation into three domains is occurring. The similarity between the enthalpies of heating and cooling curves demonstrates that no metastable state is formed. A subsequent heating scan confirms this, since it is identical to the first heating scan.

FTIR spectroscopy

Pure galactocerebrosides containing non-hydroxy fatty acids show marked spectral differences between metastable and stable forms [6]. The most notable differences are seen in the amide I and II regions of the spectrum which occur between 1700 cm^{-1} and 1500 cm^{-1}. The amide I region consists of absorptions due to C=O stretching vibrations weakly coupled to N–H bending and C–N stretching vibrations. The amide II region, on the other hand, consists of absorptions due to N–H bending vibrations strongly coupled to C–N stretching vibrations [10].

Fig. 3 shows difference spectra obtained by subtraction of the aqueous background from the spectra of hydrated quenched (metastable) and reheated (stable) galactocerebrosides. In the metastable form, the amide I band consists of a major absorption at 1646 cm^{-1} and a shoulder at 1630 cm^{-1}, whilst the amide II band is at 1546 cm^{-1}. Upon formation of the stable form, the lower amide I band in the metastable form decreases in frequency from 1630 cm^{-1} to 1615 cm^{-1}, and increases in intensity. This decrease in frequency is a result of the formation of hydrogen bonds which draw electrons from the C=O bond. The amide II band increases in frequency upon conversion to the stable form as a result of the formation of hydrogen bonds involving N–H groups in a plane perpendicular to the N–H bending [10,11].

Spectral differences are apparent in the 1200–1000 cm^{-1} region associated with C–O stretching vibrations of the galactose and sphingosine hydroxyl groups (Fig. 3b). It is not possible to assign the peaks in this region to specific C–O bonds, but decreases in frequency of the peaks at 1072, 1053 and 1023 cm^{-1} (metastable form) to 1064, 1045 and 1018 cm^{-1} (stable form) are apparent, due to a weakening of the C–O bond caused by an increase in the degree or strength of hydrogen bonding. These shifts suggest that the galactose and/or the sphingosine hydroxyl groups play a role in the formation of the polymorphic forms of the cerebroside.

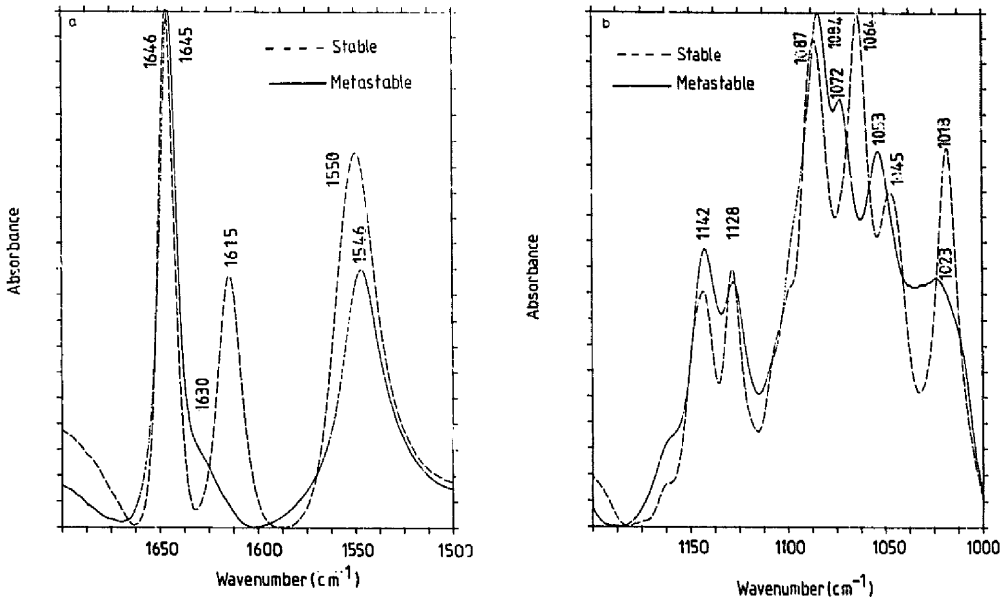

Fig. 3. FTIR difference spectra of amide region (a) and C–O stretching region (b) of type II galactocerebrosides in the metastable (———) and stable states (--- — —). Absorbance scales in arbitrary units.

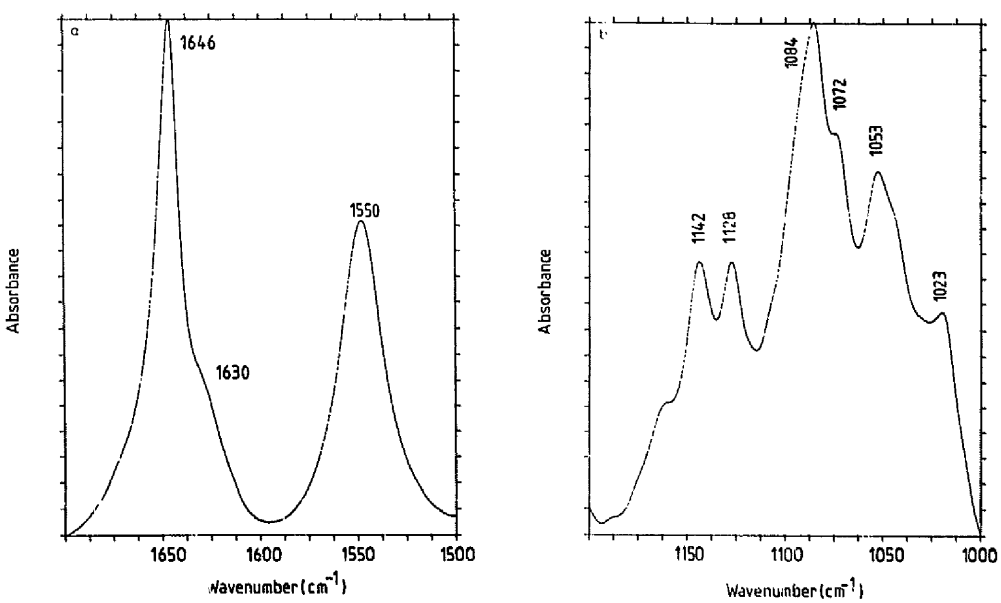

Fig. 4. FTIR spectrum of the amide region (a) and C–O stretching region (b) of anhydrous type II galactocerebrosides. Absorbance scale in arbitrary units.

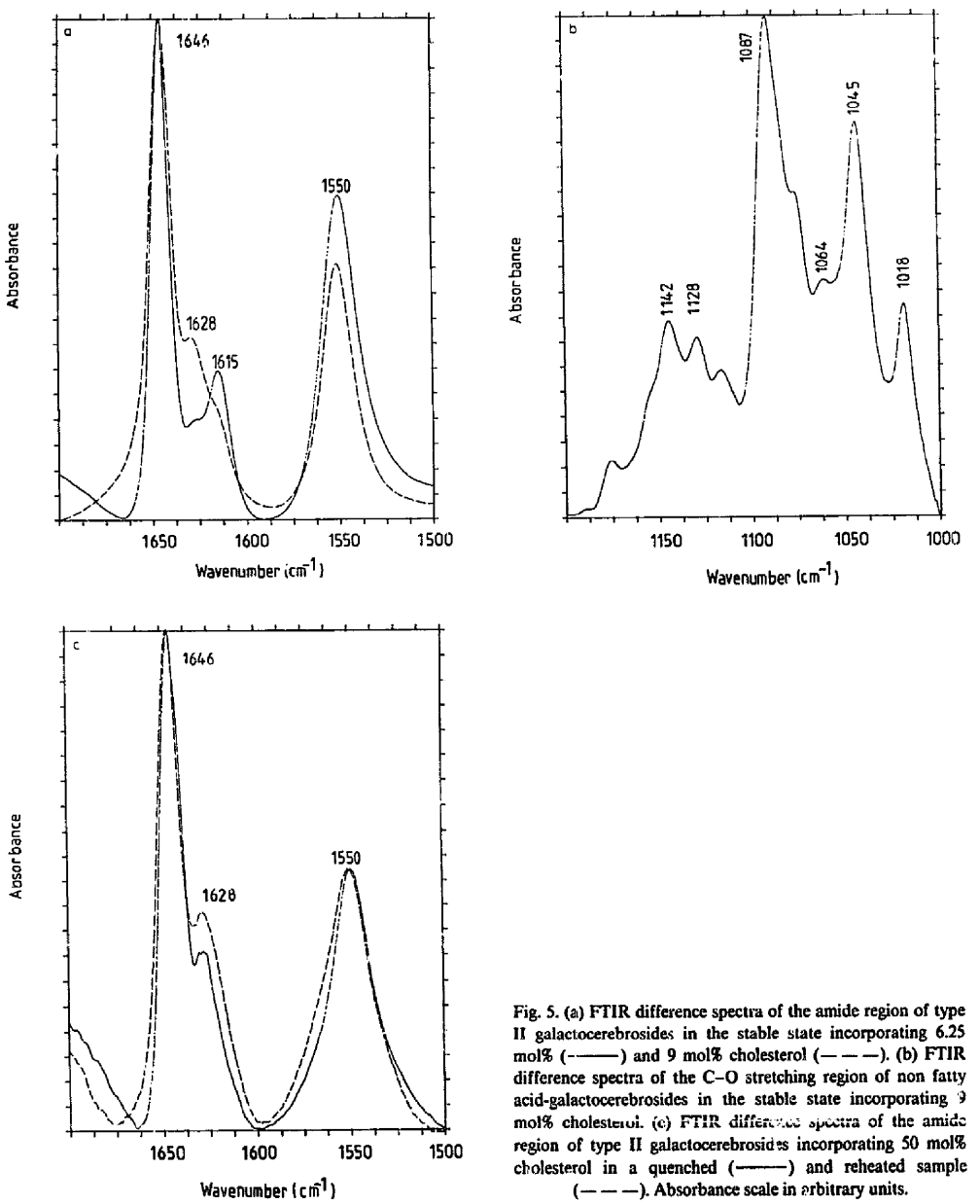

Fig. 5. (a) FTIR difference spectra of the amide region of type II galactocerebrosides in the stable state incorporating 6.25 mol% (———) and 9 mol% cholesterol (— — —). (b) FTIR difference spectra of the C–O stretching region of non fatty acid-galactocerebrosides in the stable state incorporating 9 mol% cholesterol. (c) FTIR difference spectra of the amide region of type II galactocerebrosides incorporating 50 mol% cholesterol in a quenched (———) and reheated sample (— — —). Absorbance scale in arbitrary units.

The spectra obtained from a sample of anhydrous galactocerebrosides are shown in Figs. 4a and b. The method of sample preparation did not affect the spectra. The amide II absorption of the anhydrous form occurs at 1550 cm^{-1}, the same frequency as that of the stable polymorphic

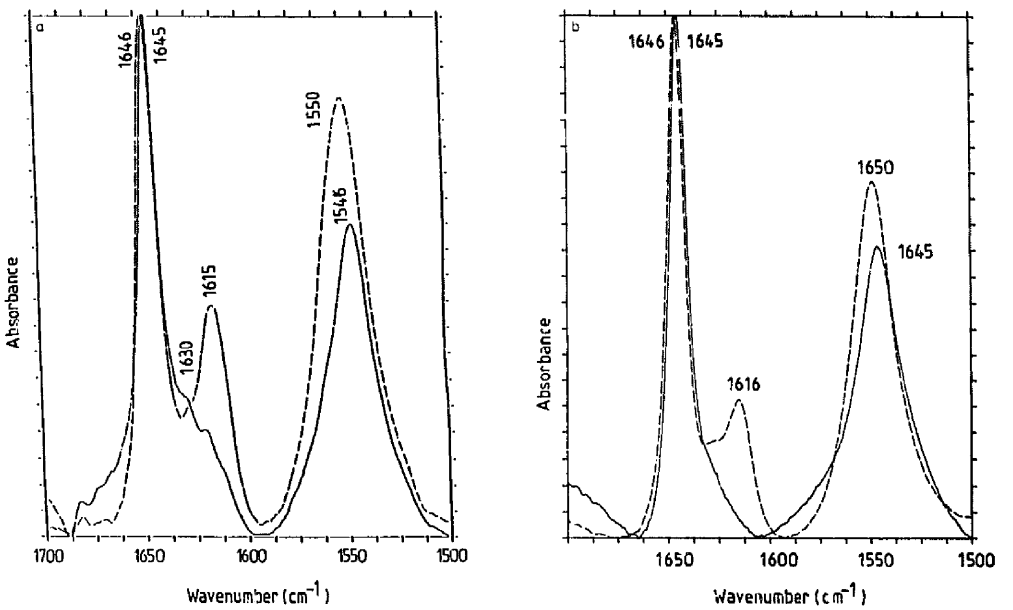

Fig. 6. FTIR difference spectra of the amide region of type II galactocerebrosides incorporating 9 mol% (a) and 33 mol% (b) DPPC in a quenched (———) and a reheated (— — —) sample. Absorbance scale in arbitrary units.

form. The spectrum of the anhydrous galactocerebroside in the amide I region and the C–O stretching region is identical to that of the metastable polymorph.

Incorporation of cholesterol into galactocerebroside bilayers has a significant effect upon the spectrum (Fig. 5). The intense amide I peak at 1615 cm^{-1} in the stable state is progressively reduced in intensity and increased in frequency as the proportion of cholesterol incorporated into the bilayer is increased. At 9 mol% cholesterol, only a shoulder at 1628–30 cm^{-1} occurs. In the C–O stretching region of the spectrum the peak occurring at 1064 cm^{-1} in the stable state is also progressively reduced in intensity with increasing cholesterol content (Fig. 5b). The effect of addition of 50 mol% cholesterol to cerebroside bilayers is shown in Fig. 5c. The amide I and II peaks occur at 1646 cm^{-1} and 1550 cm^{-1}, respectively, in both quenched and reheated samples.

The effect upon spectra of incorporating varying amounts of DPPC into cerebroside bilayers is shown in Fig. 6. At 9 and 33 mol% DPPC the amide I peaks in reheated samples occur at 1646 cm^{-1} and 1615 cm^{-1} and the amide II band occurs at 1550 cm^{-1}. At 33 mol% DPPC a slight reduction of the second amide I peak at 1615 cm^{-1} takes place. Addition of 50 mol% DPPC (not shown) results in the disappearance of the second amide I band. The amide I and II band peak positions are at 1646 cm^{-1} and 1550 cm^{-1}, respectively, in both quenched and reheated samples.

Discussion

Cerebrosides exhibit complex polymorphic phase behaviour. Earlier studies (Refs. 1–3,12,13 and references therein) and our results (in Fig. 1) show the existence of a metastable as well as a stable polymorphic form. The occurrence of one high-temperature melting endotherm at 80 °C on the first heating scan and two peaks at 74.8 °C and 78.7 °C in the subsequent heating scan must be due to phase separation occurring, associated with the heterogeneous character of the acyl chains of type II galactocerebrosides.

The FTIR difference spectra provide evidence concerning the hydrogen bonding of the stable and metastable forms. The spectra presented in Figs. 3a and b show pronounced spectral changes upon conversion from the stable to the metastable polymorphic form. In the stable cerebroside form, two distinct amide I peaks are seen. It is possible that one of the components arises from water of hydration. However, upon deuteration, both bands shift by about 15 cm^{-1} (not shown). If either band were due to water, it would be found some 400 cm^{-1} lower in ^2H_2O than in H_2O. Furthermore, in spectra recorded at 70°C, no change in either band position is seen (not shown). We therefore conclude that these two bands represent two different populations of C=O groups. The low frequency of the band at 1616 cm^{-1} suggests this population of C=O groups is more strongly hydrogen-bonded than the other population. In the spectrum of the metastable form, the second amide I absorption is only observed as a weak shoulder at around 1630 cm^{-1}.

The similarity between the spectra of the anhydrous cerebroside and the metastable cerebroside indicates that the cerebroside headgroups in the metastable and anhydrous states are in a similar hydrogen-bonding and perhaps conformational state. X-ray diffraction patterns of anhydrous and metastable galactocerebrosides have been found to be identical [3]. In addition, hydration of cerebrosides in the presence of ethylene glycol produces a state with thermotropic properties intermediate between the metastable and stable states [7,14]. This was taken as evidence of dehydration, due to decreased water activity, producing a state with thermotropic properties tending towards those of the metastable state.

Our calorimetric studies show that addition of 9 mol% cholesterol to the cerebroside has little effect upon thermograms (Fig. 1d–f). The enthalpy changes, peak temperatures and cooperativity of the phase transitions are slightly reduced due to disruption of the chain packing. In cooling scans, two peaks are seen in a complex exotherm as a cerebroside/cholesterol phase and a pure cerebroside phase solidify. The presence of an heating exotherm in a subsequent heating scan indicates that the metastable state is still formed in the presence of 9 mol% cholesterol.

Heating scans of mixtures containing 34 mol% cholesterol show the behaviour characteristic of metastability (Fig. 1i). Initial heating scans show four endotherms, the result of phase separation of cerebroside/cholesterol mixtures into four domains. The basis of this separation into so many domains is presumably related to the heterogeneity of the cerebroside sample.

The high temperature of the fourth transition suggests that the phase responsible for this endotherm does not contain cholesterol, but is pure cerebroside, perhaps of uniform cerebroside chain length and saturation. Presumably, in the presence of cerebrosides of different composition the onset temperature of chain melting of this cerebroside phase is reduced to 79°C. This phase separates almost immediately upon cooling, and gives rise to an exotherm centered at 80.6°C. However, the enthalpy change associated with this transition is small and therefore only a very small fraction of the cerebroside solidifies at this temperature. Most of the cerebroside solidifies, still mixed with cholesterol in a transition with a reduced cooperativity, such that no enthalpy change can be detected. The phase separation of this mixture is not immediate, allowing us to assume that in our FTIR experiments we have only one major phase. A minor phase contains the relatively small amount of pure cerebroside which solidified immediately upon cooling, whilst the major phase contains most of the cerebrosides and cholesterol in a homogeneous mixture. It is this second phase which dominates the spectrum.

The thermotropic behaviour of a full range of cholesterol/cerebroside mixtures have been studied [15] and no metastable transition was detected at 50 mol% cholesterol.

We can see from Fig. 6 that incorporation of cholesterol into galactocerebroside bilayers in the stable state produces marked spectral changes (these changes are not due to absorption bands arising from cholesterol). The intensity of the second amide I peak of the stable state decreases as the concentration of cholesterol increases, until at 9 mol% cholesterol only a shoulder at 1628 cm^{-1} is seen. This reduction in intensity and increase in frequency is due to disruption of hydrogen bonds involving one of the C=O group populations.

In the C–O stretching region the peak at 1064

cm^{-1} of the stable state is also progressively reduced in intensity until at 9 mol% cholesterol it is no longer seen (Fig. 5b). Cholesterol appears to disrupt hydrogen bonding involving either the galactose hydroxyls or the sphingosine hydroxyl group. The fact that only one vibrational mode of the headgroup is affected is striking and suggests that the interaction between cerebrosides and cholesterol is highly specific in nature.

Increasing cholesterol content further to 50 mol% resulted in identical spectra after quenching and reheating. The rearrangements in the hydrogen-bonding network associated with the changes in band frequency, which occurred in previous samples upon quenching, no longer occur at this cholesterol concentration.

Fig. 2 illustrates thermograms obtained from an galactocerebroside bilayers incorporating 9, 33 and 50 mol% DPPC. At 9 mol%, DPPC thermograms show endotherms with reduced enthalpy changes due to disruption of cerebroside–cerebroside interactions by DPPC. Cooling scans exhibit a much lower enthalpy change than that of heating scans due to formation of a metastable cerebroside form. The phase separation of cerebroside molecules observed in the pure lipid and in the presence of cholesterol is much reduced in the presence of 9 mol% DPPC.

Increasing the DPPC concentration to 33 mol% produced three endothermic transitions, with a further reduction in enthalpy change due to a further decrease in the cooperativity of the cerebroside transitions. As cerebrosides and DPPC exhibit gel-state immiscibility [9], it is likely that the lower of the three peaks represents the melting of a DPPC phase containing only a small amount of cerebroside, and the remaining transitions arise from processes involving two cerebroside phases, each of which contains a small amount of DPPC. A heating scan subsequent to a cooling scan shows thermotropic behaviour characteristic of a metastable cerebroside form.

The disruption by cholesterol of the cerebroside–cerebroside interactions causing a reduction in the cooperativity of the cerebroside thermal transitions was apparent from the increase in frequency and decrease in intensity of the second amide I band and the C–O band at 1064 cm^{-1} in the stable form with increasing cholesterol concentration. Due to the rapid nature of the phase separation of cerebroside and DPPC upon cooling from the liquid crystalline state, these spectral changes are not observed at 9 mol% DPPC and are barely noticeable at 33 mol% DPPC. The amount of DPPC required to disrupt the highly cooperative nature of the cerebroside thermal transitions would therefore appear to be quite low. This phase separation of cerebrosides and DPPC has also been described in studies of mixtures of DPPC and excess *N*-palmitoylgalactosylsphingosine [9], although the time-course of the phase separation in the present study is much reduced. This may be related to the use of a heterogeneous cerebroside mixture in this study.

At 50 mol% DPPC, thermograms demonstrate that no metastable form is produced by quenching. The enthalpy changes associated with heating and cooling scans were similar and no exotherms was seen in heating scans. Cooling scans indicate immediate separation of the lipids into three phases. This phase separation does not result in DPPC-free cerebroside phases, as indicated by the very low peak temperature and degree of cooperativity of heating endotherms in thermograms, and the absence of the second amide I band in spectra (not shown). The absence of metastability may be due to the presence of DPPC in the cerebroside domains and/or interfacial effects.

Conclusions

Cerebroside polymorphism is a well-documented phenomenon. It has been demonstrated by X-ray diffraction [3], DSC [1,2], Raman spectroscopy [5] and FTIR spectroscopy [6]. Our results are in general agreement with these studies, although we note formation of multiple cerebroside domains in pure cerebrosides, cerebroside/cholesterol and cerebroside/DPPC mixtures. The basis for this separation of cerebroside into multiple domains presumably lies in the heterogeneous nature of our sample, and may be a function of fatty acid chain length, fatty acid chain unsaturation, position of the double bond in unsaturated chains.

The FTIR difference spectra presented in Figs. 3 and 4 support an involvement of hydration changes and hydrogen bond network re-arrange-

ment in the interconversion between stable and metastable cerebroside forms as proposed by other workers [3,7,14], with the metastable form being a dehydrated, less strongly hydrogen-bonded form. The hydrogen-bonding scheme in the metastable form appears similar to that of the anhydrous cerebroside.

Addition of low concentrations of cholesterol to cerebroside bilayers broadens and reduces the peak temperature of thermal transitions, as has been demonstrated previously [8], as a consequence of the disruption of cerebroside–cerebroside interactions. This disruption is apparent in spectra of cerebroside/cholesterol mixtures as a decrease in intensity and an increase in frequency of the second amide I band and a decrease in intensity of the C–O stretching band at 1064 cm^{-1}.

Incorporation of cholesterol into cerebroside bilayers results in the formation of a cerebroside phase with properties intermediate between a gel state and a liquid-crystalline state. Although DSC thermograms and spectral data show behaviour characteristic of metastability, the metastability must differ from that of the pure cerebroside.

Addition of 50 mol% cholesterol to cerebroside bilayers results in the complete abolition of all metastable behaviour as detected by DSC [15]. This finding is supported by the lack of any re-arrangement of the hydrogen-bonding network involving the cerebroside headgroups (Fig. 5c).

Upon addition of DPPC to cerebroside bilayers, the same general phenomena are observed. Transitions are broadened and their peak temperatures reduced due to disruption of the bilayer. This disruption cannot be monitored spectroscopically under the conditions utilised in these experiments due to almost immediate phase-separation of cerebrosides and DPPC upon cooling (Figs. 2, 6). At 50 mol% DPPC, no thermal transitions indicative of metastable state formation were observed (Figs. 2i–j), and no rearrangement of the cerebroside hydrogen-bond network was seen.

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References

1 Freire, E., Bach, D., Correa-Freire, M., Miller, I. and Barenholz, Y. (1980) Biochemistry 19, 3662–3665.
2 Reed, R.A. and Shipley, G.G. (1987) Biochim. Biophys. Acta 896, 153–164.
3 Ruocco, M.J., Atkinson, D., Small, D.M., Skarjune, R.P., Oldfield, E. and Shipley, G.G. (1981) Biochemistry 20, 5957–5966.
4 Skarjune, R. and Oldfield, E. (1982) Biochemistry 21, 3154–3160.
5 Bunow, M.R. and Levin, I.W. (1980) Biophys. J. 32, 1007–1022.
6 Lee, D.C., Miller, I.R. and Chapman, D. (1986) Biochim. Biophys. Acta 359, 266–270.
7 Curatolo, W. (1985) Biochim. Biophys. Acta 817, 134–138.
8 Ruocco, M.J. and Shipley, G.G. (1984) Biophys. J. 46, 695–707.
9 Ruocco, M.J., Shipley, G.G. and Oldfield, E. (1983) Biophys. J. 43, 91–101.
10 Susi, H. (1969) in Structure and Stability of Biological Macromolecules (S.N. Timasheff and Fasman, G.D., eds.) pp. 573–663, Marcel Dekker, New York.
11 Bellamy, L.J. (1980) The Infrared Spectra of Complex Molecules, Chapman & Hall, London.
12 Curatolo, W. (1982) Biochemistry 21, 1761.
13 Curatolo, W. (1985) Biochemistry 24, 6608.
14 Miller, I.R. and Bach, D. (1986) Biochim. Biophys. Acta 863, 121–127.
15 Johnston, D.S. and Chapman, D. (1988) Biochim. Biophys. Acta 838, 603–614.