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Circular dichroism spectra of aqueous dispersions of sphingolipids

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The circular dichroism (CD) spectra of a number of sphingolipids dispersed in water have been studied. The lipids include cerebrosides such as palmitoyl cerebroside, glucocerebroside from the spleen of Gaucher patients, bovine brain galactocerebrosides type I and type II, (BCI and BCII, respectively) and also sphingomyelins such as egg sphingomyelin and bovine brain sphingomyelin. Changes in the CD spectra of the lipids which occur upon heating and cooling and the effects of cholesterol, phosphatidylcholine and the opiate leucine enkephalin were studied.

Introduction

Sphingolipids including glycosphingolipids and sphingomyelin are derived from the long chain aliphatic base sphingosine. Glycosphingolipids are present in almost all membranes in small amounts but large quantities occur in myelin membranes. Cerebrosides dispersed in water form lamellar arrays and exhibit a complex thermotropic behaviour, featuring hysteresis and form metastable as well as stable rigid crystalline polymorphs [1–4]. It is believed that this behaviour is caused by head group interactions with concomitant change in their hydration [3–5] which then affects the subsequent packing of the hydrocarbon chains. Some evidence to support this contention has been obtained from NMR studies [5,6], which show that the hydrogen bonded network is altered during the transition between the different rigid crystalline phases, causing subsequent changes in the hydrocarbon chain packing. To obtain more detailed information on the nature of the interpolar group interaction, one has to identify the residues within the polar domain which are responsible for these interactions. One significant feature which distinguish cerebrosides and sphingolipids from the phospholipids is the amide bond by which the acyl chain is attached to the sphingosine base. Amide groups are known to form strong hydrogen bonds with each other and with other proton donors and acceptors. Furthermore amide residues give characteristic infrared (IR) and Raman spectra modulated by the nature and the extent of this hydrogen bonding. The amide groups also absorb in the ultraviolet (UV) region at approx. 200 nm. Being adjacent to an asymmetric carbon atom optical rotary dispersion (ORD) and circular dichroism (CD) spectra dependent upon the orientation of the amide groups in the sphingolipids is to be expected, and indeed some CD spectra of sphingomyelin vesicles have been measured [7]. In this paper we present CD spectra of some sphingolipids, their variation with the change in the state of the lipid as affected by temperature, by added dipalmitoylphosphatidylcholine (DPPC) or cholesterol and by interaction with the opiatepeptide leucine enkephalin.

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Materials and Methods

Sphingolipids. Four cerebrosides and two sphingomyelin lipids have been investigated. The cerebrosides which were investigated are: palmitoyl cerebroside (PGC), glucocerebroside from the spleen of Gaucher patients (GGC), type I bovine brain galactocerebroside containing 2-hydroxyacyl chains (BCI), type II bovine brain galactocerebroside (BCII), containing no hydroxyl residues in the acyl chain. The sphingomyelins investigated are from chick eggs containing primarily palmitic acid (ESM), and from bovine brain containing predominantly stearic acid (BBSM). All the sphingolipids were purchased from Sigma.

CD measurements. For the measurements the sphingolipids were dispersed in 5 mM pH 7.5 phosphate buffer by sonication with a tip sonicator or in a bath sonicator. The suspensions of the mixtures of the sphingolipids with DPPC or with cholesterol were prepared by mixing of chloroform-methanol solutions of the lipids, evaporation of the solvents in N_2 and in vacuum, and then sonication of the dry lipids in the buffer solution. The lipid concentration in the aqueous dispersion varied between 1 and 2 mg/ml. The CD measurements were performed in Kings College, University of London, using a JASCO CD spectrometer and cells with pathlengths of 0.2 and 0.5 mm.

Results

In Figs. 1–5 are shown the CD spectra of some cerebrosides and sphingomyelin lipids in aqueous dispersions at room temperature, modified by heat treatment, by cholesterol and by interaction with enkephalin. The observed CD spectra feature a broad low positive band between 210 and 240 nm, with its peak varying between 210 and 230 nm which in some cases disappears. Their most characteristic extremum is the negative one varying between 207 and 198 nm with a half width of 10–20 nm. A positive extremum of large amplitude varied between 195 nm to below 185 nm beyond the instrumental capability. The CD spectra have some similarities to the CD spectra of proteins and polypeptides [7–9] where the arrangement of the amides in the polar plane is either random or in a β-sheet type conformation.

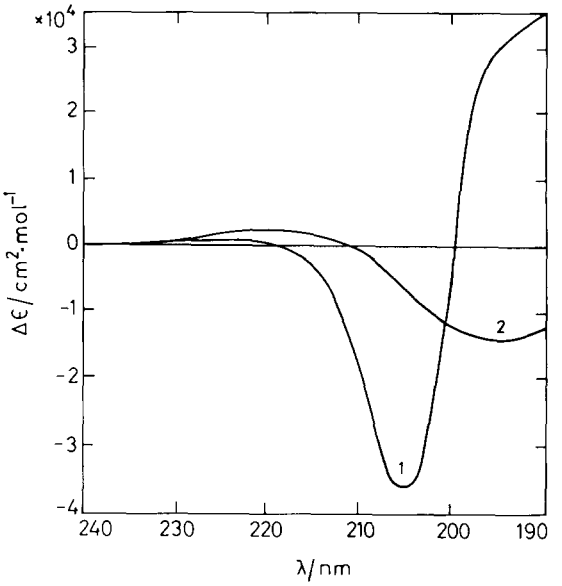

Fig. 1. CD spectra of a BCI lipid dispersion containing cholesterol (brain cerebroside fraction with 2-hydroxyacyl chains) at a molar ratio of nearly 0.5 cholesterol per BCI. Curve 1 after dispersion in aqueous buffer at room temperature. Curve 2 after heating the dispersion to 70°C and cooling.

The CD spectra of various different cerebrosides were measured, these include: PGC, GGC, BCII and BCI lipids. The first three cerebrosides are known to show at temperatures below 50°C a metastable rigid-crystalline state, obtained by rapid cooling of the lipid from the liquid-crystalline state, in addition to a stable one [3–5]. The metastable state is known to transform into the stable form very slowly at room temperature. The transition rate increases with temperature and above 60°C it is completed within a minute. The BCI lipid containing 2-hydroxyacyl chains does not, however, have any metastable states and shows no hysteresis.

When dispersed at room temperature the BCI lipid shows large elipticities with λ_m^- at 205–206 nm and even a larger positive extremum at $\lambda_m^+ =$ 193 nm, besides a broad shallow elipticity band around 220 nm. The shape of the spectrum is retained but its intensity is strongly reduced when heated above 50°C and cooled back to room temperature. Above the phase transition temperature, the CD spectrum vanishes and only a small

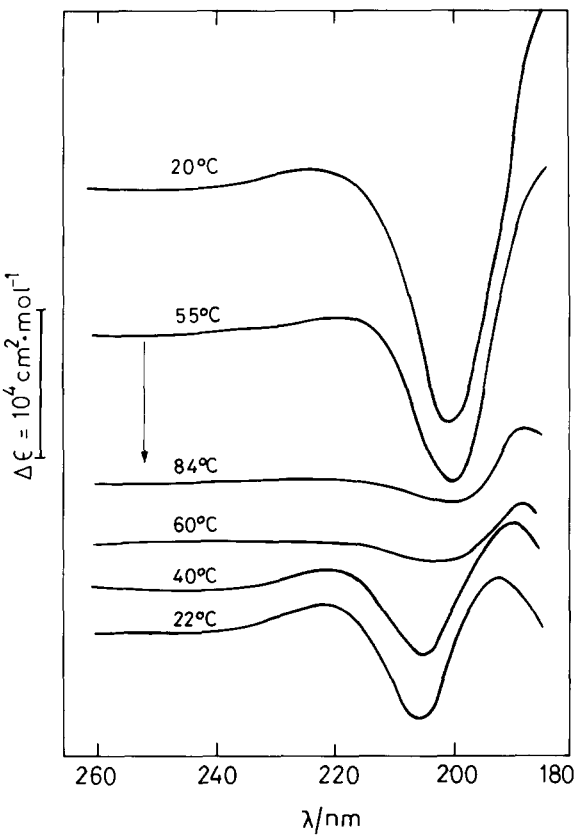

Fig. 2. CD spectra of BCII (brain cerebroside with non hydroxyacyl chains) in course of a heating-cooling cycle. The cycle proceeds in the direction of the arrow starting from the top.

fraction of it recovers after cooling to room temperature.

The presence of cholesterol in the lipid has been studied and it is observed that the addition of cholesterol, enhances the elipticity but does not alter the λ of the extrema in the spectra. Incubation of the aqueous dispersion at 50°C or above causes a shift of the peaks to lower wave lengths with their concomitant broadening. In Fig. 1 the CD spectra of the cerebroside BCI, containing cholesterol at a molar ratio of about 2.2 BCI per cholesterol are presented after dispersion at room temperature and after consecutive heating to 70°C and cooling. The presence of cholesterol augments the negative extremum and for a molar fraction of cholesterol = 0.3 it is $\Delta\varepsilon = 3.6 \cdot 10^4$ cm^2/mol compared to a value of $\Delta\varepsilon = 8 \cdot 10^3$ for the BCI lipid alone. A change in the shape of the peak occurs with a broadening and a shift toward lower wave lengths upon heating and cooling. Dipalmitoylphosphatidylcholine is known to enter as a spacer between the cerebroside molecules and to disrupt their cooperative structure [10,11]. In the present experiments DPPC shifts the negative extremum of BCI toward lower wavelengths with concomitant broadening of the band both in non-heated and in heated dispersions.

Different sphingolipids behave differently upon heating. While the Gaucher cerebroside and the sphingomyelins when heated above their phase-transition temperature retain most of their ellipticity, the BCI, BCII and palmitoyl cerebrosides lose it completely, recovering it only in part upon cooling. In Fig. 2 the CD spectra of the cerebroside BCII in a heating-cooling cycle is presented. Note the shift in the peak wavelengths and the change in the relative intensities of the different extrema after completion of the heating-cooling cycles. The CD spectra of BII, when dispersed at room temperature, show a negative extremum at 200 nm of about $\Delta\varepsilon = 1.5 \cdot 10^4$ cm$^2 \cdot$ mol^{-1} and a broad positive dichroism of $\Delta\varepsilon = 10^3$ cm$^2 \cdot$ mol^{-1} at 225 nm. Heating above the phase transition reduces the intensity of the spectrum to about 10% of its original value. Lowering the temperature brings back the spectrum but changes its shape. The negative peak is shifted to 206 nm and its intensity is about $5.5 \cdot 10^3$ cm$^2 \cdot$ mol^{-1}. The intensity of the positive peak at 220 nm remains about $1.1 \cdot 10^3$ cm$^2 \cdot$ mol^{-1} and the low-wavelength positive peak is shifted to 192 nm. Storing overnight at room temperature intensifies the spectra. Addition of cholesterol produces spectra with peaks shifted toward higher wavelengths when dispersed at room temperature. Heating the suspensions causes a shift of the spectra toward lower wavelengths.

Palmitoyl cerebroside, when dispersed at room temperature shows a negative peak in the CD spectrum at 202 nm. Upon heating the lipid dispersion to 85°C and then cooling the peak shifts to 205 nm. Upon reheating to 65°C and cooling it λ_m shifts to 201 nm. The CD spectrum is very weak at 65°C and practically disappears at 85°C. The CD spectrum of Gaucher's cerebroside seems to be unaffected by the heating cycle. Its negative

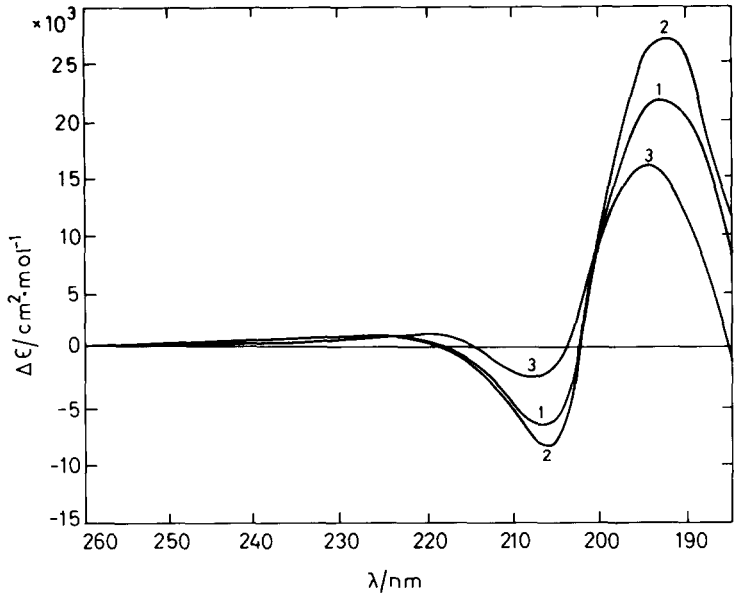

Fig. 3. CD spectra of a pure dispersion of BCI (curve 1) and after addition of leucine enkephalin. Curve 2, amide ratio of enkephalin to BCI 0.65; curve 3, amide ratio 1.45.

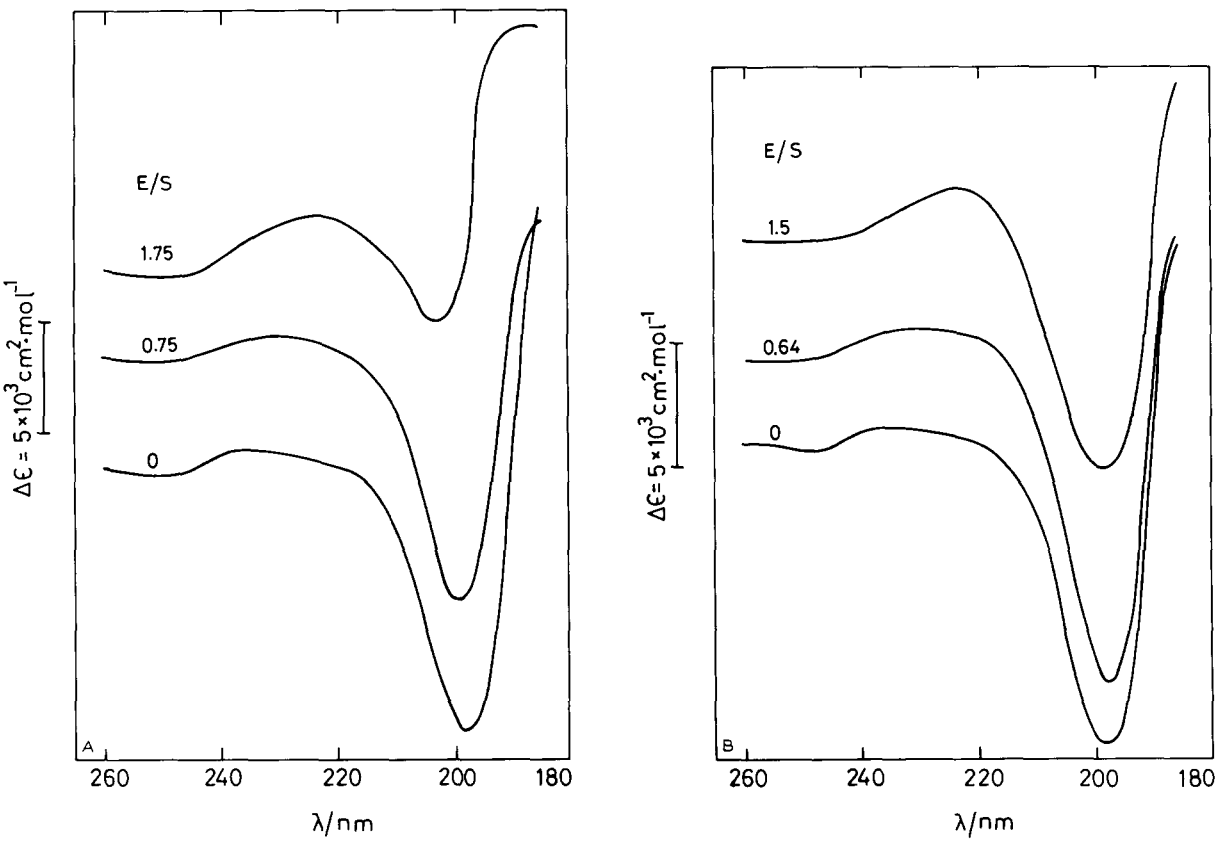

Fig. 4. (A) CD spectra of pure chick egg sphingomyelin dispersions and upon their interaction with enkephalin. The enkephalin to sphingosine amide ratios are indicated on the curves. (B). CD spectra of bovine brain sphingomyelin dispersions and upon their interaction with enkephalin. The enkephalin to sphingomyelin amide ratios are indicated on the curves.

peak is always at 200–201 nm. Its spectrum is retained at high temperature. DPPC and cholesterol up to molar fractions of 0.3 have only a small effect on the spectra at room temperature. Heating of the suspensions and subsequent cooling causes a small shift of their peak toward shorter wave lengths.

Sphingomyelins; Chick egg and bovine brain sphingomyelins have identical spectra with a negative extremum $1.2 \cdot 10^4$ $cm^2 \cdot mol^{-1}$ at 198 nm. The same spectrum is retained upon heating the sphingomyelins above their phase-transition temperature.

Effect of leucine enkephalin

Leucine enkephalin which is an opioid pentapeptide, shows no CD spectrum by itself or in the presence of phosphatidylcholine or phosphatidylethanolamine lipids. It does, however, have an effect on the CD spectra of the cerebroside BCI. As shown in Fig. 3 at a molar ratio of 0.55 enkephalin amide per cerebroside, it intensifies the CD spectrum while at higher molar ratios it weakens it. First an enhancement and then a lowering of the elipticity occurs indicating that the incorporation of enkephalin into the surface amide array takes place at low concentrations. At higher concentrations the enkephalin disrupts the cooperative amide conformation. This behaviour is also observed, with the spingomyelin lipids.

The effect of leucine enkephalin on the two sphingomyelins is illustrated in Figs. 4A and 4B. Addition of increasing concentrations of enkephalin up to an equivalent ratio (amides of enkephalin to sphingomyelin) of 0.4 enhances the negative CD extrema. Upon further increase of the molar ratio one observes a consecutive decrease of the negative extremum with its shift toward higher wavelengths. The adverse effect of enkephalin in the two concentration regions seems to be common to cerebrosides and to sphingomyelins. In the presence of an excess of enkephalin the height of the broad positive ellipticity of sphingomyelins in the wavelength region between 245 and 210 nm is increased and its peak is gradually shifted toward lower wavelengths. This last effect seems to be unique to the sphingomyelin.

Discussion

Comparison can be made between the CD spectra observed with the sphingolipids and those which occur with the amino acids of proteins. The chirality of the sphingosine, which determines the optical rotatory dispersion and the circular dichroism, is opposite to that of the natural amino acids. Taking this into account and trying to interpret the sphingo-lipid CD spectra with the aid of basic spectra [9], one could conclude that besides random and sheets and turns, an appreciable portion of the amides may be in a helical conformation. It is however difficult to visualise how the amide groups restricted to the polar plane could form a helix.

For an adequate conformational analysis, an independent determination of the detailed structure of the polar region is required. X-ray diffraction studies carried out on single crystals, but not on aqueous dispersions, could provide information useful for such a structural analysis. The question is to what extent the structure determined in an almost dry single crystal is preserved after dispersion of the lipid in water. This may be so in the case of the BCI lipid and possibly also in the case of the other cerebrosides when dispersed in water at room temperature. The x-ray diffraction of single crystals of the BCI lipid has been investigated and analysed by Pascher and Sundell [12]. They found that the lateral hydro-carbon packing is of a hybrid type. Zig-zag chains with parallel planes are arranged in pleated sheets. These sheets contain alternately either fatty acid or sphingosine chains which have a mutually almost perpendicular chain plane orientation. The planes of the sugar rings are nearly parallel to the surface, forming together with the polar ceramide part a lateral network of inter- and intramolecular hydrogen bonds. At the same time the NH group gives intramolecular hydrogen bonds, mainly with the hydroxyl group of the fatty acid. However, since the planar rigid amide groups adopt a perpendicular orientation towards the axes of the two hydrocarbon chains [13], it also forms parallel pleated sheets with alternating orientation of its plane. The difference in the angle of the amide planes in the consecutive sheets is 99° and 81°, respectively. This β-sheet structure is in good

agreement with the CD spectra of BCI dispersed at room temperature.

Studies by other workers show that upon extensive hydration and upon heating, the hydrogen bond network disintegrates, the carbohydrate rings protrude into the aqueous solution [14], and the pleated sheet conformation falls apart.

Consistent with this is the dramatic weaking of the CD spectrum of galactocerebroside BCI when hydrated and of the other cerebrosides when heated above their transition temperature. In the non-hydroxy acyl cerebrosides as seen by infrared spectroscopy [16], the amides seem to be directly involved in intermolecular hydrogen bonding. This gives rise to different relative orientations of the amides, affecting the intermolecular interactions. These interactions are probably the reason for the observed polymorphism, hysteresis and existence of metastable states. This is reflected in the changes in the CD spectra of the BCII lipids which occur during the heating and cooling cycle, Fig. 2. The amide related polymorphism reveals itself also in the changes in the wavelengths of the extrema and in the elipticities of the cerebrosides without the hydroxyl group in the acyl chain when cooled rapidly from 85°C (metastable) or cooled after reheating to 70°C (stable). However, the detailed CD and IR spectra in the metastable and in the stable states of the different non-hydroxy cerebrosides are not identical for all cerebrosides. The detailed dependence and its conformational significance in individual cases is under further investigation.

Studies made by other workers using calorimetry and X-ray diffraction of cerebroside-cholesterol dispersions indicate a marked tendency for strong cerebroside-cerebroside intermolecular associations to occur which can exclude cholesterol. This is true particularly with dispersions at low temperatures. At high temperatures, the cerebroside hydrogen bond matrix is disrupted and the cholesterol molecules are solubilised within the lipid structures to form a homogeneous mixed cerebroside/cholesterol liquid-crystal phase [15]. This may explain the changes we observe in the CD spectra (see Fig. 1) where a broadening of the spectrum occurs after heating to temperatures of 50°C and higher.

Our studies show that the brain opiates enkephalins interact very strongly with the amide groups of the sphingomyelins and the cerebrosides as seen by the effect on their CD spectra. We have chosen for these experiments the pentapeptide leucine enkephalin since it is too short to have any CD spectrum of its own and all the measured CD spectra can be attributed to the sphingolipids and to any resultant 'complexes'. The low ratios of enkephalin to sphingolipid which we have used are relevant to the possible interactions prevailing in the brain. At these same ratios the enkephalin are seen to enhance the CD spectra of the sphingolipids indicating their integration into the lipid network. We do not consider that this enkephalin interaction with sphingolipids is directly related to their biological function. It may be, however, that the sphingolipid head groups form a pathway for the enkephalin to reach the specific receptors involved. The sphingolipid-enkephalin interaction could also regulate the availability of the receptors if these are embedded in a sphingolipid-rich membrane.

Further studies of the amide group of sphingolipids including gangliosides are in progress using CD and IR spectroscopy.

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