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# FTIR spectroscopic analysis of the amide and acid bands of ganglioside $G_{M1}$, in pure form and in mixtures with DMPC

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The amide I bands of sphingolipids show complicated patterns due to intra- and intermolecular interactions via hydrogen bonds. In order to assign the amide I absorption bands of the ganglioside $G_{M1}$ to the different amide groups in the headgroup and back bone, the compounds *N*-acetylgalactosamine, *N*-acetylneuraminic acid, glucocerebroside and ceramide III were examined as reference systems. The frequencies of the COOH and $COO^-$ bands of the sialic acid residue of $G_{M1}$ were determined by pH-titration and were found to absorb at 1729 $cm^{-1}$ and 1605 $cm^{-1}$, respectively. In $D_2O$ the three amide groups of $G_{M1}$ give one broad absorption band at 1627 $cm^{-1}$, whereas in the glucocerebroside intra- and intermolecular interactions of the amide group give rise to three distinct amide I bands. For a solid sample of $G_{M1}$ in KBr also one broad band was observed in the amide I region. We also studied the influence of the ganglioside $G_{M1}$ on model membranes of DMPC as host lipid. The change of the $CH_2$ stretching vibrational absorption bands as a function of temperature reveal that addition of $G_{M1}$ to DMPC leads to increased phase transition temperatures $T_m$ with increasing ganglioside content. No $Ca^{2+}$ binding to the $COO^-$ group of $G_{M1}$ was observed.

## 1. Introduction

Gangliosides, sialic acid containing glycosphingolipids, are involved in a variety of cell surface phenomena in the central nervous system where they are particularly abundant. Their influence on model membranes has been investigated by various methods such as differential scanning calorimetry [1,2], EPR spectroscopy [3], $^1H$-, $^{13}C$-NMR spectroscopy [4] and electron microscopy [5]. In order to elucidate the molecular order of the ganglioside $G_{M1}$ in model membranes, we examined DMPC/$G_{M1}$ mixtures by FTIR. Two aspects of the $G_{M1}$ molecule are particularly important in characterizing its influence on the host membranes. Changes in the absorption bands (amide I and amide II region) of the functional groups of the voluminous headgroup of $G_{M1}$ (four neutral and one negatively charged sugar residues) reflect the interaction at the water/membrane interface. The interpretation of these bands requires their unequivocal assignment to the functional groups present in the oligosaccharide moiety of $G_{M1}$. Detailed investigations of the amide vibrational modes have often been performed on polypeptides where, due to the sensitivity of the amide I frequency to secondary structure, it is possible to distinguish between $\alpha$-helical or $\beta$-sheet conformations [6]. In our work, the band assignment of the amide frequencies belonging to the NANA and GalNAc moieties in the headgroup is complicated by the additional band of the amide function of the ceramide residue as well as by the vibrational band of the $COO^-$ group of the sialic acid, which also absorbs in this frequency region. Therefore, the components of $G_{M1}$, i.e., *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid (NANA), glucocerebroside (GlcCer) and ceramide III (CerIII) were studied as reference systems.

Furthermore, the temperature-dependent $CH_2$ stretching vibrations give information about the phase state of the membrane. The maxima of these absorption bands are typically shifted by about 2–3 $cm^{-1}$ to higher values at the phase transition temperature due to an increase of disorder of the fatty acid chains.

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Abbreviations: FTIR, Fourier transform infrared; ATR, attenuated total reflection; $G_{M1}$, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; NANA, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; GlcCer, glucocerebroside; CerIII, ceramide III.

## 2. Materials and Methods

$G_{M1}$ was either extracted from bovine brain according to Svennerholm and Fredman [7] or bought from Biosynth (Staad, Switzerland) (used for DMPC/$G_{M1}$ mixtures). GlcCer (from human spleen), CerIII (without $\alpha$-hydroxy fatty acids), GalNAc, NANA and DMPC were purchased from Sigma (Deisenhofen, Germany) and used without further purification.

*Sample preparation*

Transmission spectra of $G_{M1}$, GlcCer, GalNAc and NANA were recorded using solutions or dispersions of 10 mg of the compounds in 200 $\mu$l $D_2O$ in a 50 $\mu$m $CaF_2$ cell. The pH dependent measurements of $G_{M1}$ and NANA were carried out by mixing the same solution with increasing volumes of 1 M DCl or 1 M NaOD. For spectra of the dry compounds as KBr-pellets about 2 mg of the sample per 100 mg KBr were used.

For ATR measurements of pure $G_{M1}$ 50 $\mu$l of a $10^{-2}$ M solution of $G_{M1}$ in $CHCl_3$/MeOH (2:1 v/v) were spread on an ATR crystal (ZnSe). The solvent was evaporated and the film was dried for 2 h under reduced pressure. Hydration was achieved by placing a drop of $H_2O$ on the crystal mount, covering the mount with a thermostated lid which was then heated to 40°C for at least half an hour. Mixtures of DMPC/$G_{M1}$ were prepared by mixing $10^{-2}$ M solutions ($CHCl_3$/MeOH (2:1, v/v)) of each sample in the desired ratio. The successive preparation steps were the same as described above.

Transmission spectra were measured at room temperature. The temperature-dependent ATR measurements were carried out by recording a spectrum at 2 C° intervals between 6°C to 40°C.

For each spectrum 512 interferograms were accumulated using a Bruker IFS 48 FTIR spectrometer. The spectral resolution was 2 $cm^{-1}$.

Deconvolution and simulation of IR bands was performed using a home-written program applying the deconvolution procedure described by Kauppinen et al. [8]. A resolution enhancement factor (K factor [9]) of approx. 2.5 was used.

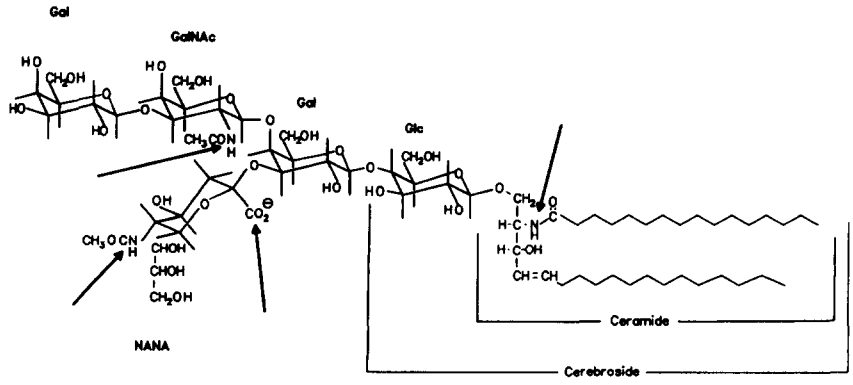
For bandshape simulations a non-linear least-squares fitting procedure was applied using the Levenberg-Marquardt algorithm [10]. Frequencies were determined by self-deconvolution and used as fixed parameters for the fitting procedure.

## 3. Results and Discussion

For polypeptides it is well documented that the frequencies of the amide I and II bands are dependent on the secondary structure formed by the peptide in aqueous solution. Using a set of proteins with known $\alpha$-helical, $\beta$-sheet and random-coil content procedures have been developed to determine the secondary structure of proteins in solution whose X-ray structure is not known [11,12].

Much less is known about the sensitivity of the amide I frequencies on the extent of intermolecular interactions in lipids with amide groups. Sphingolipids are abundant as membrane lipids and the thermotropic behaviour of sphingomyelin and gluco- and galactocerebrosides has been well characterized by DSC [1,13]. Only few investigations of cerebrosides using IR spectroscopy have been performed and it was shown that the observed amide I frequencies depend on the phase state of the lipids [14,15].

For gangliosides the situation is even more complicated, as in addition to the amide group of the ce-




Ganglioside $G_{M1}$

Fig. 1. Molecular structure of $G_{M1}$. The amide groups of the headgroup and the back bone as well as the carboxyl group are marked with arrows.

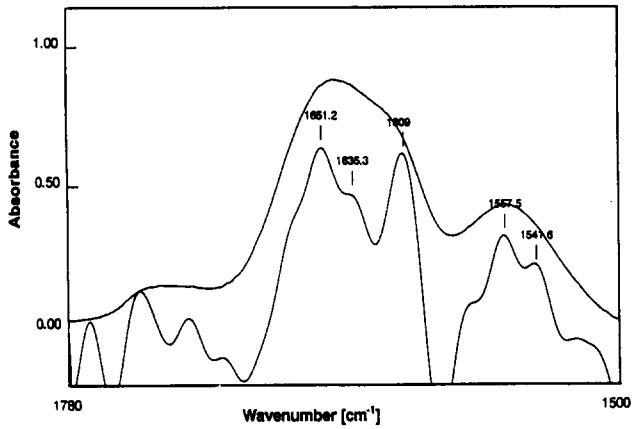



Fig. 2. Original (top) and deconvolved (bottom) spectrum of dry $G_{M1}$ (KBr pellet) in the spectral region from 1780 $cm^{-1}$ to 1500 $cm^{-1}$. A resolution enhancement factor of 2.5 was used.

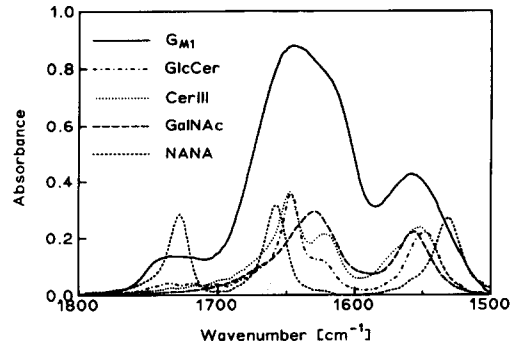



Fig. 3. IR spectra of $G_{M1}$ and the related model compounds (KBr pellets) in the spectral region from 1800 $cm^{-1}$ to 1500 $cm^{-1}$.

ramide backbone two amide groups are present in the oligosaccharide headgroup, namely those of the GalNAc and the NANA residues (see Fig. 1). Further complications arise from the presence of the carboxyl group of the sialic acid. This group can either be protonated or deprotonated giving rise to different vibrational bands, the one for the negatively charged form ($COO^-$) being in the frequency region of the amide I bands.

Our primary goal was to assign the observed vibrational bands to the different amide groups in the headgroup and backbone and to the carboxyl group, using spectra of NANA, GalNAc, GlcCer and CerIII as references.

The compounds were first investigated in the dry state as KBr pellets. The spectrum of $G_{M1}$ in KBr reveals three major absorption bands in the region from 1800 to 1500 $cm^{-1}$. The band at 1731 $cm^{-1}$ arises from the C=O stretching vibration of the COOH group of the sialic acid. The two other bands are the amide I and the amide II band. Each of these is a sum of several overlapping bands as shown in the deconvolved spectrum (Fig. 2). In order to assign these bands to the different functional groups of $G_{M1}$, spectra of related compounds were recorded and compared to the $G_{M1}$ spectrum. The reference spectra and the vibrational frequencies are shown in Fig. 3 and Table I. The spectra of NANA and GalNAc reveal only one amide I peak at 1656 $cm^{-1}$ and 1629 $cm^{-1}$, respectively. The shift in wavenumber might stem from the influence of different hydrogen bonds although both molecules have the same *N*-acetyl group. In the case of NANA there is probably an interaction of the amide group with the adjacent glycerol residue. The amide I region of GlcCer and CerIII show two bands, probably due to species with different intermolecular or intramolecular hydrogen bonding. An unequivocal assignment of the bands of the reference compounds to the deconvolved bands of $G_{M1}$ is difficult because the amide I frequencies are sensitive to the crystal structure. In the $G_{M1}$ spectrum the vibrational bands are much broader than in the reference compounds, indicating that the molecules are disordered. This can be understood by the complicated chemical structure and the high molecular weight of $G_{M1}$. In contrast, the vibrational bands of GalNAc, NANA, GlcCer and CerIII are relatively sharp. Despite these differences in crystal packing it is clear that the 1727 $cm^{-1}$ band of NANA may be assigned to the protonated carboxyl group (see below). In $G_{M1}$ the same band is found at 1731 $cm^{-1}$. The major maxima of the amide band of $G_{M1}$ after deconvolution are at 1651 $cm^{-1}$ and 1609 $cm^{-1}$ with an indication of another possible band at 1635 $cm^{-1}$. The 1609 $cm^{-1}$ is too low in frequency to be assigned to an amide I band. We will show (see below) that this band is clearly due to the unprotonated carboxyl group. The remaining bands are thus amide I bands from the head group and the ceramide backbone.

In polypeptides, the amide II bands are less sensitive to intermolecular interactions and changes in secondary structure and are normally not analyzed. This is similar in our lipid reference compounds. Despite the appearance of two amide I bands in the spectra of dry GlcCer and CerIII only one amide II band is observed

TABLE I

*Characteristic absorption bands of $G_{M1}$ and related model compounds in the region of 1800–1500 $cm^{-1}$, measured in KBr*

The wavenumbers were determined by Fourier self-deconvolution.

| Sample | Vibrational mode | | | |
|---|---|---|---|---|
| | $\nu$(CO) COOH ($cm^{-1}$) | $\nu$(CO) amide I ($cm^{-1}$) | $\nu(COO^-)$ $COO^-$ ($cm^{-1}$) | $\delta$(NH), $\nu$(CN) amide II ($cm^{-1}$) |
| $G_{M1}$ | 1731 | 1651, 1635 | 1609 | (1575), 1557, 1541 |
| NANA | 1727 | 1656 | | 1530 |
| GalNAc | | 1629 | | 1556 |
| GlcCer | | 1646, 1621 | | 1547 |
| CerIII | | 1647, 1620 | | 1550 |

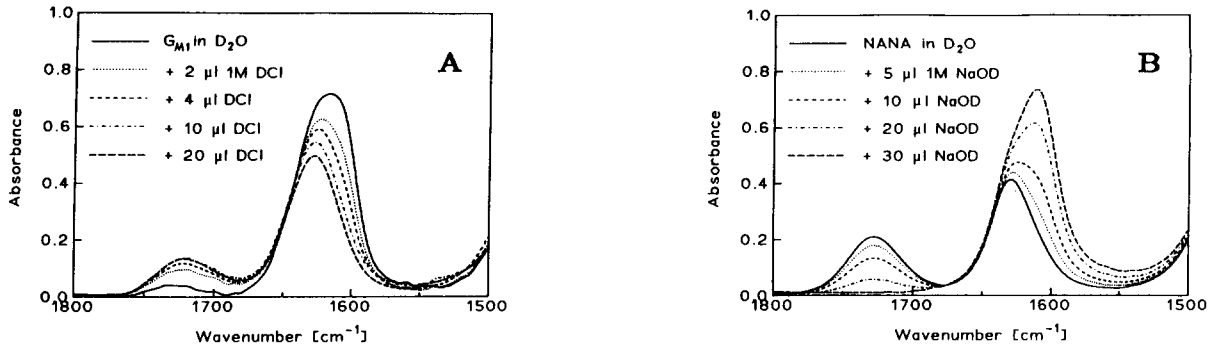



Fig. 4. pH dependence of the amide I vibrational band of $G_{M1}$ (A) and NANA (B).

in both cases. In the spectrum of dry $G_{M1}$ deconvolution gives rise to two amide I and at least two amide II components. Again, an unambiguous assignment to the amide groups of the headgroup or the backbone is not possible.

The frequency of the amide I band is sensitive to hydration of the amide group. This can be shown by dissolving model compounds, such as *N*-methyl- or *N*-ethylacetamide in water and unpolar solvents. For instance, a shift of the amide I frequencies from 1653 cm$^{-1}$ in $CCl_4$ to 1623 cm$^{-1}$ in $D_2O$ and from 1648 cm$^{-1}$ to 1617 cm$^{-1}$, respectively, is observed. In addition, small differences in amide I band frequencies are found when $D_2O$ instead of $H_2O$ is used as a solvent. The amide II vibrational band is mainly due to an N-H deformation vibration with an admixture of the C-N stretching mode. A change in solvent from $H_2O$ to $D_2O$ shifts this band to lower wavenumbers because of

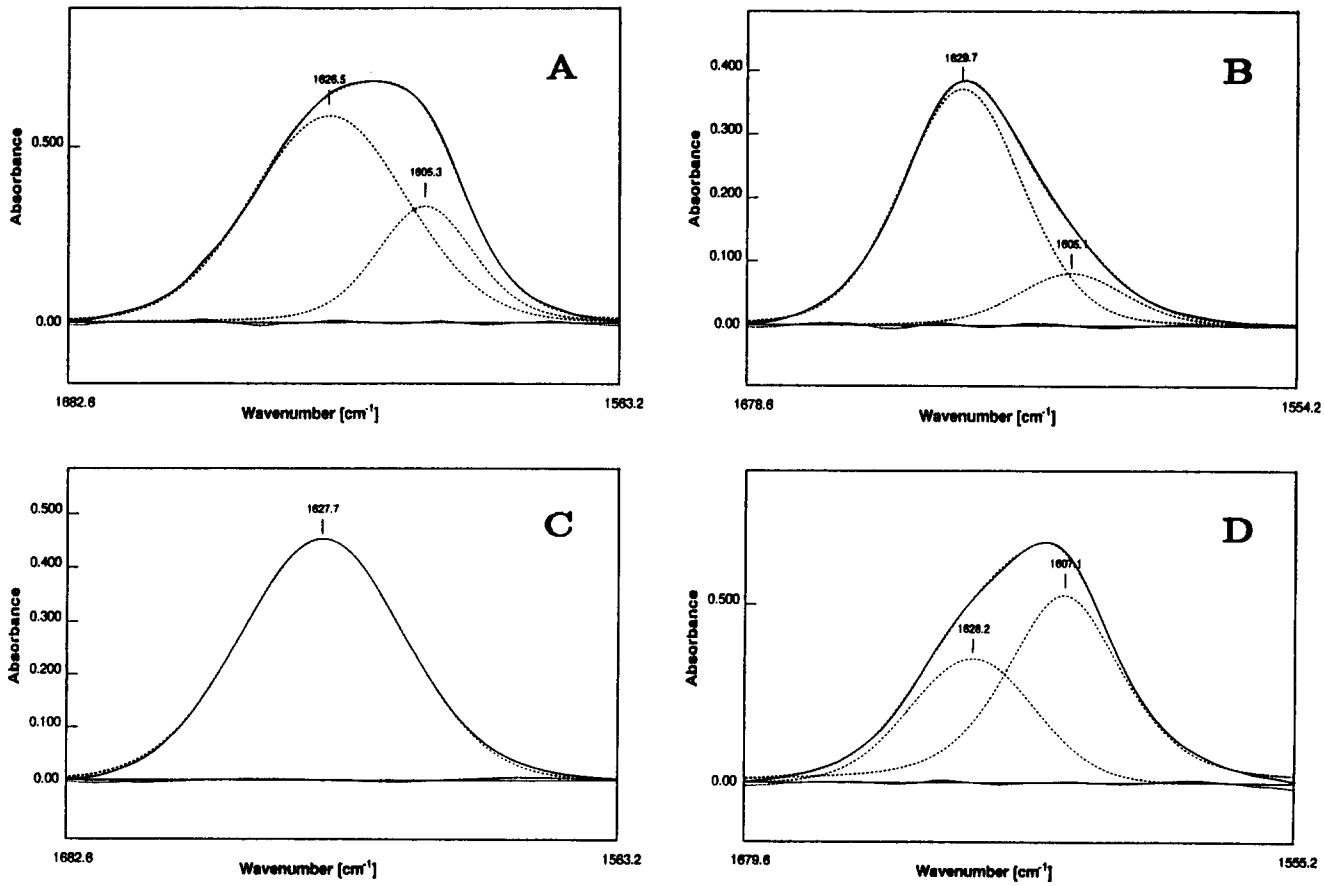



Fig. 5. Measured (full lines) and fitted (dashed lines) spectra of $G_{M1}$ (A: dissolved in $D_2O$, C: after addition of 20 µl DCl) and of NANA (B: dissolved in $D_2O$, D): after addition of 30 µl 1 M NaOD). The dotted lines are the differences of the fitted and measured spectra.

the vibrational isotope effect. This occurs only in these cases where the N-H proton is completely exchanged by deuterium.

$G_{M1}$, dissolved in water, is in a micellar state because of the large, well hydrated headgroup and does not form bilayers [16]. The spectrum of $G_{M1}$ in $D_2O$ is shown in Fig. 4A. It is clearly different from the spectrum in the dry state (Fig. 2). As expected, the amide II band has shifted to wavenumbers below 1500 $cm^{-1}$ because of complete H/D exchange at the amide groups. The intensity of the band at 1729 $cm^{-1}$ is relatively low indicating that upon dissolving $G_{M1}$ in pure $D_2O$ most of the sialic acid groups are in a deprotonated state. Protonation can be achieved by addition of DCl and leads to a shift of the apparent maximum of the amide I band to higher values. This effect is accompanied by a change in bandshape and a decrease of intensity of this band coupled with an increase of intensity of the COOH band (Fig. 4A). Protonation is evidently complete after addition of 10 $\mu$l 1 M DCl. A determination of the pH of the micellar solution was not possible due to the low sample volume. Our goal was only to show, that the 1729 $cm^{-1}$ band can be assigned to the COOH group and the shoulder at 1605 $cm^{-1}$ is caused by the $COO^-$ moiety.

A similar behaviour supporting our assignment was observed for the vibrational bands of NANA dissolved in $D_2O$, only that in this case the carboxylic group is mostly in the protonated state after dissolution, as the intensity of the residual band at 1605 $cm^{-1}$ is low. Complete deprotonation can be achieved by addition of 30 $\mu$l of 1 M NaOD as then the 1729 $cm^{-1}$ band, charasteristic for the protonated form has disappeared (Fig. 4B).

The spectra of $G_{M1}$ and NANA were deconvolved and then fitted with Gaussian-Lorentzian band shapes to determine the exact band frequencies. This is shown in Fig. 5. After addition of DCl only one amide I band is observed in $G_{M1}$, centered at 1627 $cm^{-1}$ (Fig. 5C). In pure $D_2O$ the band shape can be simulated by using just two component bands, again the first one due to the amide I band at 1627 $cm^{-1}$ and the second due to the $COO^-$ group at 1605 $cm^{-1}$ (Fig. 5A). Essentially the same band frequencies are observed for NANA (Figs. 5B, 5D). In the deprotonated form the intensity of the amide I band is much smaller for NANA as only one amide group is present in the molecule compared to three in the case of $G_{M1}$.

The appearance of only one amide I band in $G_{M1}$ at 1627 $cm^{-1}$ is evidence for extensive hydrogen bonding of all three amide functions with the $D_2O$ molecules. The frequency at 1627–1630 $cm^{-1}$ seems to be characteristic for amide groups in contact with water and without other specific intermolecular interactions via hydrogen bonds. The same frequency is found for GalNAc dissolved in $D_2O$ (see Table II). Acquotti et al. [17] as well as Scarsdale et al. [18] performed $^1$H-NMR studies of $G_{M1}$ in solutions of DMSO-$d_6$ and DMSO-$d_6$/$D_2O$ (98:2, v/v), respectively. They suggested interresidual interactions in the $G_{M1}$ molecule via hydrogen bonds between the N-H proton of GalNAc and the $COO^-$ group of NANA. As a consequence the environments of the amide carbonyl groups of GalNAc and NANA should be different. Whether these interactions are also present in pure $D_2O$ as solvent cannot be decided on the basis of our IR spectra, because of the equilibrium between protonated and deprotonated state of the $COO^-$ group on the one hand, and because of the interactions of the amide groups with $D_2O$ molecules on the other hand. The frequency of the $COO^-$ vibration is the same for NANA and $G_{M1}$. This frequency is possibly insensitive to the nature of the hydrogen bond donor.

TABLE II

*Characteristic absorption bands of $G_{M1}$ and related model compounds in the region of 1800–1600 $cm^{-1}$, measured in $D_2O$*

The wavenumbers were determined by Fourier self-deconvolution.

| Sample | Vibrational mode | | |
|---|---|---|---|
| | $\nu$(CO) COOH ($cm^{-1}$) | $\nu$(CO) amide I ($cm^{-1}$) | $\nu(COO^-)$ ($cm^{-1}$) |
| $G_{M1}$ | 1729 | 1627 | 1605 |
| NANA | 1729 | 1630 | 1605 |
| GalNAc | | 1627 | |
| GlcCer | | 1647, 1629, 1611 | |

In contrast to these findings for the water soluble compounds NANA, GalNAc and $G_{M1}$ as a micellar solution, three overlapping amide I bands at 1647, 1629 and 1611 $cm^{-1}$ are observed for the glucocerebroside in $D_2O$ dispersion (Table II). This lipid forms lamellar bilayers at room temperature. The different amide I bands are evidently caused by different types of intermolecular interactions between neighbouring lipid molecules and between lipid and water. That strong lipid–lipid interactions via amide hydrogen bonds are present is also indicated by the restricted H/D exchange, i.e., the slow disappearance of the amide II band at 1545 $cm^{-1}$ when spectra are recorded as a function of time after addition of $D_2O$ (not shown). Apparently the amide N-H group is involved in a strong intermolecular hydrogen bond. A reference spectrum of CerIII in $D_2O$ could not be recorded, as CerIII is neither soluble in $D_2O$ nor can it be hydrated due to the lack of a hydrophilic headgroup.

We finally investigated the behaviour of $G_{M1}$ in mixed bilayers with DMPC as a host phospholipid. The influence of $G_{M1}$ on the phase behaviour of DMPC can be monitored by following the frequency of the $CH_2$

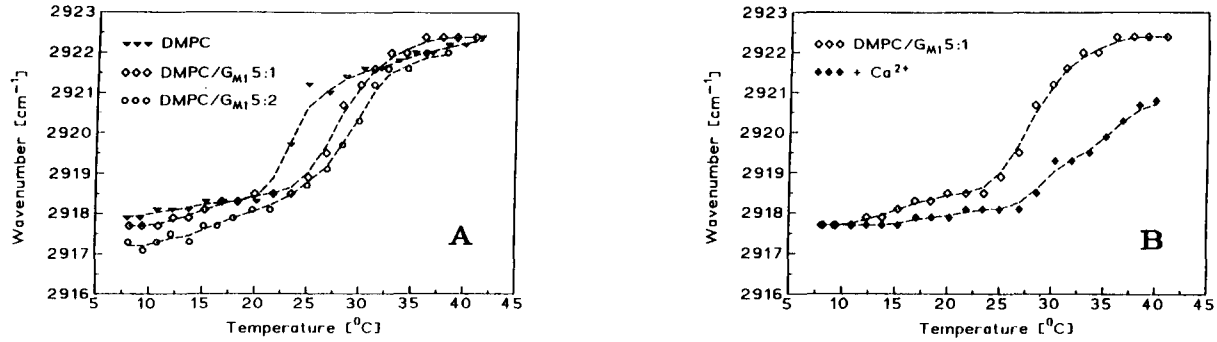



Fig. 6. Temperature dependence of the antisymmetric $CH_2$ stretching vibration of DMPC and DMPC/$G_{M1}$ 5:1 and 5:2 mixtures (A) and DMPC/$G_{M1}$ 5:1 before and after $Ca^{2+}$ addition (B).

stretching vibrational bands as a function of temperature. The maxima of the $CH_2$ stretching vibrational bands shift to higher wavenumbers, when the bilayer passes into the $L_\alpha$ phase. Addition of $G_{M1}$ to DMPC leads to increased phase transition temperatures with increasing ganglioside content (Fig. 6A). The transition temperatures are 23.3°C for DMPC, 28.4°C for DMPC/$G_{M1}$ (5 : 1, mole/mole) and 29.5°C for DMPC/$G_{M1}$ (5 : 2, mole/mole). This effect, indicating a stabilization of the DMPC bilayer by $G_{M1}$, is accompanied by a broadening of the phase transition due to a decreased number of cooperatively melting lipid molecules. Our results agree with these of Sillerud et al. [2] and Ollmann [19]. Hinz et al. [4], using sonicated vesicles observed a decrease of the transition temperature after $G_{M1}$ addition. These contrary effects may be caused by different fatty acid chain lengths, by the different degree of saturation of the chains in the $G_{M1}$ molecules and/or by the different physical state of sonicated vesicles as compared to multilamellar systems. The amide I bands are essentially unchanged compared to the pure $G_{M1}$ micellar form.

We also studied the effect of the addition of $Ca^{2+}$ ions to these mixed bilayers. Addition of $Ca^{2+}$ to a DMPC/$G_{M1}$ 5:1 mixture results in a further shift of the transition temperature to 32.1°C (Fig. 6B). The frequencies of the $CH_2$ stretching vibrations are lowered indicating tighter packing of the acyl chains. $Ca^{2+}$ is able to bind to the sialic acid residue of $G_{M1}$ as well as to the phosphate group of DMPC. Its binding constant to these different groups has been shown to be of the same order of magnitude [9]. In the DMPC/$G_{M1}$ mixture the absorption band of the $COO^-$ vibration reveals no measurable change when $Ca^{2+}$ is added (not shown) while the asymmetric phosphate vibrational band of DMPC shows a shift to higher frequencies. In the spectrum of pure DMPC this band is changed in a similar way after $Ca^{2+}$ addition (Fig. 7A). This may indicate that in mixed bilayers of DMPC/$G_{M1}$ the binding affinity of $Ca^{2+}$ to the negatively charged phosphate group of DMPC is higher than to the sialic acid residue of $G_{M1}$. Sillerud et al. [20] and Harris et al. [21] gave NMR evidence of a chelate-like structure of the oligosaccharide moiety of pure $G_{M1}$ in the micellar form in the presence of monovalent or trivalent cations. Trivalent cations, such as $Eu^{3+}$ are apparently chelated by the hydroxyl group of the saccharide residues [20]. An influence of $Ca^{2+}$ on the $COO^-$ band of pure $G_{M1}$ and of DMPC/$G_{M1}$ bilayers was not observed by IR spectroscopy. Thus we conclude that $Ca^{2+}$ is mainly in the vicinity of the phosphate groups in DMPC/$G_{M1}$ and not bound to $G_{M1}$.

The frequency shift of the asymmetric phosphate vibrational band to higher values is apparently caused by a partial dehydration of the DMPC headgroup by

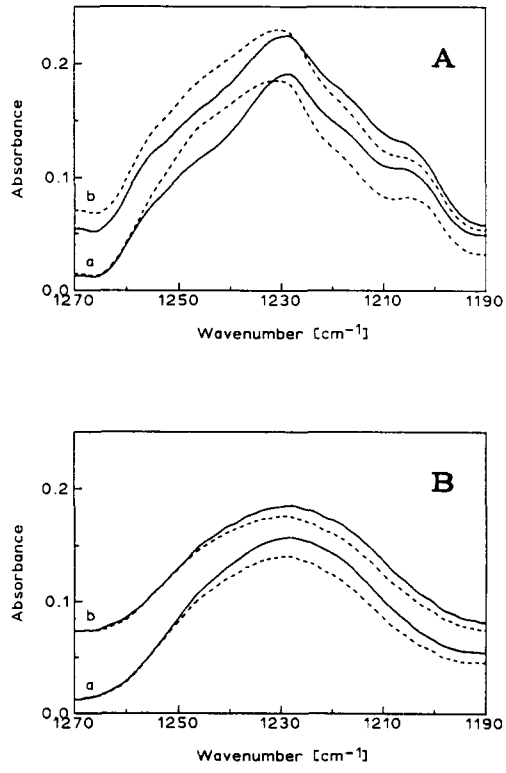



Fig. 7. The influence of $Ca^{2+}$ on the asymmetric phosphate stretching vibration of DMPC and a DMPC/$G_{M1}$ 5:1 mixture at 10°C (A) and 50°C (B). Full lines: DMPC (a) and DMPC/$G_{M1}$ (b), dashed lines: DMPC/$Ca^{2+}$ (a) and DMPC/$G_{M1}$/$Ca^{2+}$ (b).

$Ca^{2+}$. This effect is weaker at high temperatures in the liquid-crystalline phase (Figs. 7A, 7B) because then an increased penetration of water molecules into the headgroup region is possible. The dehydration is also the reason for the increase of the transition temperature after addition of $Ca^{2+}$ to the DMPC/$G_{M1}$ mixture.

## Summary

FTIR spectroscopy was used to study the amide I band of the ganglioside $G_{M1}$ by comparison with suitable reference compounds. In $D_2O$ all three amide I bands of $G_{M1}$ overlap, essentially having the same frequency at 1627 $cm^{-1}$. This indicates extensive hydration of all amide groups, including that of the ceramide backbone. The bands at 1729 $cm^{-1}$ and 1605 $cm^{-1}$ belong to the protonated and deprotonated carboxyl groups of the sialic acid residue of the headgroup. Their relative intensities change with pH. Thus the protonation state at this residue can be directly determined from the IR spectra. The line shapes in the region of the amide and $COO^-$ bands in DMPC/$G_{M1}$ are unchanged compared to pure $G_{M1}$, even after addition of $Ca^{2+}$. $Ca^{2+}$ binding to the DMPC phosphate group seems to be stronger than to the sialic acid group of $G_{M1}$.

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