*Biochimica et Biophysica Acta* 903 (1987) 78–88
Elsevier

BBA 73681

# A study of the structure of polymyxin B-dipalmitoylphosphatidylglycerol complexes by vibrational spectroscopy

Yves Babin, Jacynthe D'Amour, Marie Pigeon and Michel Pézolet

*Centre de recherche en sciences et ingénierie des macromolécules, Département de chimie, Faculté des sciences et de génie, Université Laval, Cité Universitaire, Québec G1K 7P4 (Canada)*

(Received 24 April 1987)

Key words: Polymyxin B; Dipalmitoylphosphatidylglycerol; Fourier transform infrared spectroscopy; Raman spectroscopy; X-ray diffraction

**The effect of the antibiotic polymyxin B on dipalmitoylphosphatidylglycerol (DPPG) bilayers has been studied by Raman and infrared spectroscopies and small-angle X-ray diffraction. Each polymyxin B molecule binds five DPPG molecules at physiological pH and induces a macroscopic phase separation of the complex rather than a lateral phase separation. Below the phase transition of DPPG/polymyxin B bilayers, the results obtained show that the intermolecular vibrational coupling is high and suggest that the acyl chains of the bound lipid are interdigitated and that the hydrophobic tail of the antibiotic does not penetrate this tight assembly. On the other hand, the phase transition of DPPG is shifted down from 41°C to 37°C in the complexes and remains highly cooperative. Above the phase transition of the complexes, the conformation of the acyl chains of DPPG is slightly more disordered as a result of the penetration of the polymyxin chain, but the structure of the glycerol backbone of the lipid does not seem to be affected. However, the rotational rate of the lipid appears to be restricted by the peptide.**

## Introduction

Polymyxin B is an antibiotic composed of a polycationic cyclic heptapeptide of a tripeptide tail carrying a small acyl chain [1]. Under physiological conditions, this molecule has five residues of diaminobutyric acid that are positively charged. Polymyxin B is known to increase the permeability of cell membrane towards carbohydrates [2,3] and potassium ions [4]. Because of its amphiphilic character, polymyxin B can be regarded as a very simple model to study lipid–protein interactions.

It is generally accepted that polymyxin B does not bind to zwitterionic phospholipids such as phosphatidylcholines [1,3,5], although at high concentration the peptide has a significant effect on the gel to liquid-crystalline phase transition of dimyristoylphosphatidylcholine (DMPC) liposomes [6]. On the contrary, studies on model systems have revealed a high affinity of polymyxin B for acidic phospholipids [3,5,7,8]. Interactions between this antibiotic and phosphatidic acids have been extensively studied [6–12] and a model for these interactions has been proposed by Hartmann et al. [8]. According to this model, polymyxin is able to bind up to five molecules of phosphatidic acid [8] and, due to elastic distortions of the lipid membrane, a domain of polymyxin-bound lipids may be formed in a cooperative way, depending on the pH and ionic strength. This domain exhibits an heterogenous structure

Correspondence: M. Pézolet, Département de chimie, Faculté des sciences et de génie, Université Laval, Cité Universitaire, Québec, Canada, G1K 7P4.

with an inner core in which strong electrostatic and hydrophobic interactions between the peptide and the lipid molecules are present, surrounded by an annular ring that is characterized by hydrophobic interactions only. The respective volume of the inner core and of the ring is strongly dependent on the ionic strength for a complex at a given molar ratio [9].

Interactions between polymyxin B and dipalmitoylphosphatidylglycerol have not been very much studied and no model for these interactions has been proposed yet. However, the following facts are known about the complex formed by DPPG and polymyxin B. (1) One polymyxin molecule binds five DPPG molecules and in contrast to phosphatidic acid, this binding is not cooperative [5,7]. (2) Polymyxin B seems to affect the phase transition temperature of DPPG, but there is a discrepancy among the published results. Fluorescence polarization experiments have shown that in the presence of excess lipid, DPPG-polymyxin complexes melt at 35 °C, as compared to 41 °C for pure DPPG at pH 7 [7,13]. On the other hand, two transitions at 42.2 °C and 44.5 °C were observed by differential scanning calorimetry (DSC) [14]. (3) A lateral phase separation between bound and free DPPG was reported from fluorescence polarization experiments in excess lipid conditions [7,13], but at an incubation molar ratio ($R_i$) lower than 15 molecules of DPPG per molecule of polymyxin B a precipitate was obtained. As opposed to phosphatidic acids, only one kind of bound lipid has been proposed. (4) Finally, X-ray experiments have shown that polymyxin B causes a significant reduction of the lamellar repeat distance and likely induces interdigitation of DPPG bilayers in the gel phase [15].

In the present study, we have investigated the effect of polymyxin B on the infrared and Raman spectra of DPPG in order to gain more information on the structure of this system, and at the same time, to elucidate the situation regarding the discrepant reports concerning the phase transition temperature of the lipid in the complex. Vibrational spectroscopy proved to be very effective to investigate the molecular organization of phospholipid-polypeptide complexes [16,17] since it provides useful information about particular portions of the molecules, and does not require the addition of any probe that could possibly perturb the studied system.

## Materials and Methods

### *Materials*

Dipalmitoylphosphatidylglycerol was obtained from Avanti Polar-Lipids Inc. (U.S.A.) while polymyxin B sulfate was purchased from Sigma Chemical Co. (U.S.A.). Both products were used without further purification.

To prepare samples for Raman and infrared measurements, a 4% by weight lipid dispersion in 100 mM phosphate buffer (pH 7.5) was submitted to four or five cycles of heating (55 °C)-vortex shaking-cooling (20 °C). The required amount of a 1% solution of polymyxin B in the same buffer was then added to the lipid dispersion and this mixture was submitted to the thermal treatment mentioned above. For Raman experiments, the sample was transferred into a glass capillary tube in which it was centrifuged. The white pellet was used for the measurements. For infrared measurements, the pellet was transferred, when hot, between two $CaF_2$ windows with a 6 $\mu$m mylar spacer.

### *Raman measurements*

Samples were irradiated with the 514.5 nm line of an argon ion laser (Spectra-Physics Inc., model 165) at about 150 mW power at the sample. Spectra were recorded with a computerized spectrometer (Spex Industries Inc., model 1400) described elsewhere [18] at a spectral resolution of 5 $cm^{-1}$. The monochromator was calibrated with a neon discharge lamp, and the frequencies cited later are believed to be accurate to $\pm 2$ $cm^{-1}$ for sharp peaks.

Spectra of complexes were computer corrected for solvent and polypeptide contributions by subtracting the corresponding spectra multiplied by the appropriate normalization factor. For polymyxin B, subtraction was performed by cancelling the phenylalanine band around 1005 $cm^{-1}$ in the resulting difference spectrum. If needed, spectra were corrected for fluorescence backgrounds by subtracting appropriate polynomial functions [18].

*Infrared measurements*

Spectra were recorded with a Bomen DA3-02 Fourier-transform infrared spectrophotometer with a mercury-cadmium-telluride detector and a KBr beamsplitter. 500 scans were routinely taken with a maximal optical retardation of 0.5 cm, and Fourier transformed to yield a resolution of 2 $cm^{-1}$.

Spectra in the C-H stretching region were corrected for the water background by subtracting appropriate polynomial functions. Carbonyl region spectra were first corrected by subtracting the solvent and water vapor spectra and then Fourier deconvolved using a 18 $cm^{-1}$ wide Lorentzian line and a $K$ factor of 2.2 [19].

*Small-angle X-ray measurements*

Appropriate volumes of the DPPG and the polymyxin B solution were introduced with a syringe into an X-ray thin-walled glass capillary. Capillaries were sealed and centrifuged many times to ensure proper mixture.

X-ray diffraction experiments were performed with a Warhus-Statton camera, using nickel filtered Cu K$\alpha$ line ($\lambda = 0.154$ nm) at an anode loading of 40 kV and 25 mA. The photographic plates were placed at 32 cm from the sample, in an evacuated enclosure. Exposure times were typically of the order of 1–3 days.

## Results

*Raman spectroscopy*

Fig. 1 shows the Raman spectra at 25 °C of a solution of polymyxin B (A), of a pure DPPG dispersion (B), and of a DPPG-polymyxin B complex at $R_i = 5$ (C), before (solid line) and after (dashed line) correction for the polypeptide contribution, in the acyl chains C-C (990–1200 $cm^{-1}$) and C-H (2750–3050 $cm^{-1}$) stretching mode regions. As seen on this figure from the relative intensity of the phenylalanine band near 1005 $cm^{-1}$, there is little spectral interference of the polypeptide in the C-C stretching mode region. On the other hand, it is obvious that there is an overlap between the spectra of polymyxin B and DPPG in the C-H stretching mode region. In order to eliminate the contribution of the polypeptide, we have subtracted the spectrum of polymyxin B from that of the complex using the 1005 $cm^{-1}$ phenylalanine band as an internal intensity standard for both the C-C and C-H stretching regions. All spectra and temperature profiles shown later were corrected for the peptide contribution.

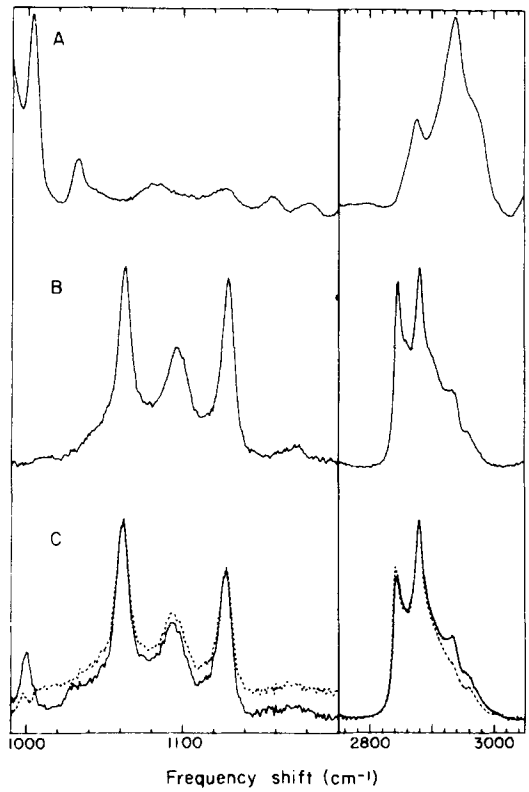


Fig. 1. Raman spectra at 25 °C of polymyxin B (A), DPPG (B) and DPPG-polymyxin B at $R_i = 5$ (C) corrected (dashed line) and not corrected (solid line) for the polypeptide contribution.

Fig. 2 shows the spectra in the two regions of interest of a pure DPPG dispersion (solid line) and of a DPPG-polymyxin B complex at $R_i = 5$ (dashed line) below and above the phase transition temperature of the lipid. For both spectral ranges, heating above the phase transition temperature or adding polymyxin B produce significant changes in the spectrum of DPPG that can be followed through well characterized intensity ratios. The C-C stretching mode region provides direct information about the intramolecular order of the acyl chains of phospholipids [20–22]. A parameter of interest in this region is the intensity ratio of the band near 1090 $cm^{-1}$ to the one near 1125 $cm^{-1}$

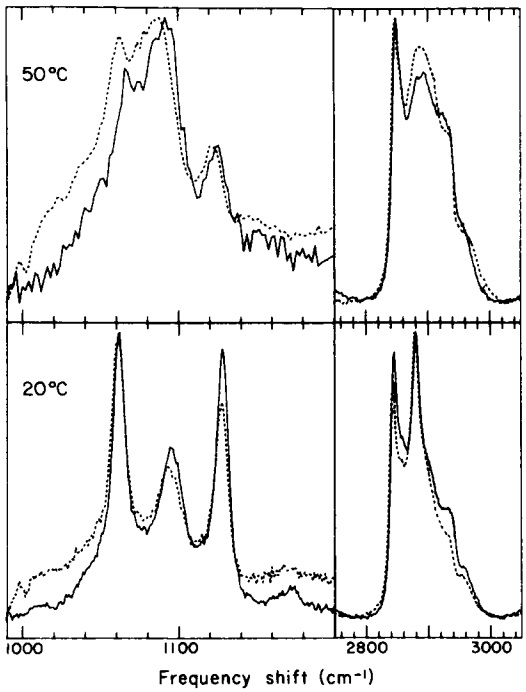


Fig. 2. Raman spectra of DPPG (solid line) and DPPG-polymyxin B at $R_i = 5$ (dashed line) at 20 ° C and 50 ° C.

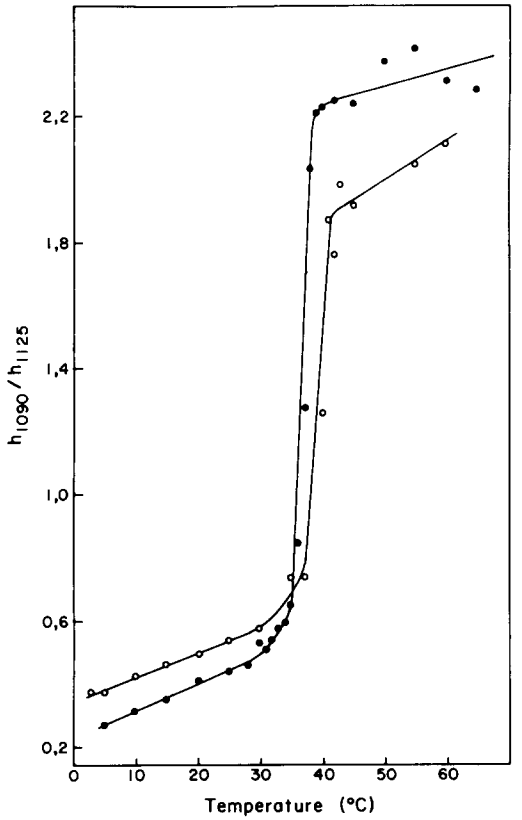


Fig. 3. Temperature profiles of DPPG (open circles) and DPPG-polymyxin B at $R_i = 5$ (full circles) derived from the $h_{1090}/h_{1125}$ peak height intensity ratio.

assigned to stretching vibrations of C-C bonds with *gauche* or *trans* conformation, respectively. Since this ratio increases with the growing population of *gauche* conformers, it can be used as a sensitive probe of the intramolecular order of the acyl chains in the bilayer.

Fig. 3 illustrates the effect of temperature on the $h_{1090}/h_{1125}$ ratio for both a pure DPPG dispersion and a DPPG-polymyxin B complex at $R_i = 5$. As seen on this figure, the gel to liquid-crystalline transition of DPPG remains highly cooperative in the presence of polymyxin B but its mid-point temperature is lowered from 41 ° C to 37 ° C. In the gel phase, the antibiotic does not seem to affect significantly the conformation of the acyl chains of DPPG. However, in the liquid-crystalline phase, even though the curves are not well defined, it seems that polymyxin decreases the intramolecular order of the lipid acyl chains. This effect can be associated with the penetration of the tail of the polypeptide in the hydrophobic core of the bilayer.

The C-H stretching mode spectral region is also very sensitive to the molecular order within the bilayer. Two intensity ratios are commonly used to follow the effect of temperature on this region of the Raman spectra of phospholipids. The first parameter that we used is the intensity ratio of the methylene antisymmetric stretching band near 2880 $cm^{-1}$ to the methylene symmetric stretching band near 2845 $cm^{-1}$. Increasing temperature results in a significant lowering of this intensity ratio and this effect has been related to the reduction of the interchain Fermi interaction, which produces an underlying broad band around 2880 $cm^{-1}$ [23], and to the increase of the acyl chain rotational mobility [24]. Therefore, this intensity ratio may be used to monitor the intermolecular vibrational coupling of the acyl chains. For example, the addition of calcium ions to dimyristoylphosphatidic acid [25] or of poly(L-lysine) to DPPG [26] causes a marked increase of this ratio which was assigned to an increase of the

intermolecular vibrational coupling due to the change of the tilt angle of the acyl chains. Similarly, O'Leary and Levin [27] have demonstrated that interdigitation of the acyl chains in lipid bilayers also causes an increase of the $h_{2880}/h_{2845}$ spectral index. Fig. 4 shows the temperature dependence of this parameter for pure and polymyxin bound DPPG. These curves, as those derived from the C-C stretching mode region, show unambiguously that the phase transition of the lipid in the complex occurs at 37°C. In addition, Fig. 4 reveals that over the entire studied temperature range, the interchain vibrational coupling is higher in polymyxin bound DPPG than in pure DPPG bilayers.

The second spectral parameter measured from the C-H stretching region is the ratio of the intensity of the band due to the methyl symmetric stretching vibration near 2930 $cm^{-1}$ to the intensity of the methylene antisymmetric stretching band near 2880 $cm^{-1}$. Increasing the temperature of the lipid bilayer increases this intensity ratio because of the apparent increase of the methyl symmetric stretching near 2930 $cm^{-1}$ due to the appearance of an underlying infrared active methylene stretching mode becoming Raman allowed as the low temperature chain symmetry is lowered, and also from a change in the Fermi resonance between the asymmetric C-H stretching mode of the terminal methyl groups and the first overtone of the symmetric $CH_2$ bending mode [28]. Therefore, this parameter can be used as a probe of the overall disorder of the lipid acyl chain matrix [29,30].

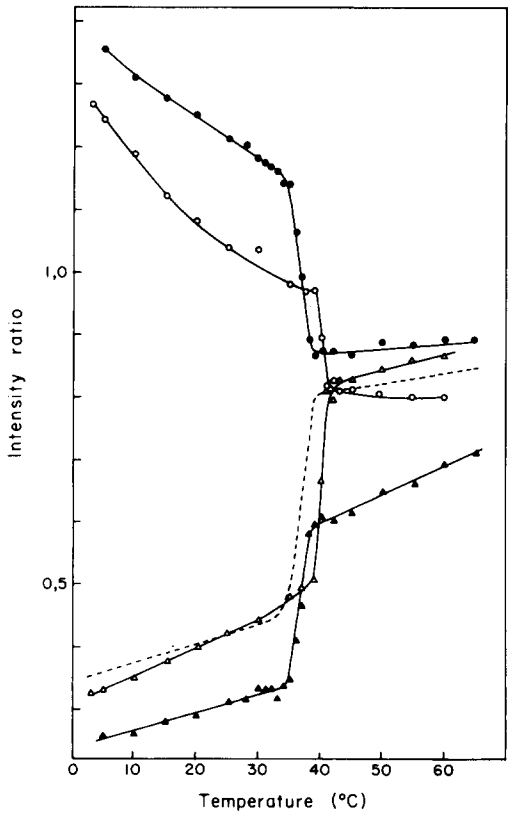


Fig. 4. Temperature profiles of DPPG (open symbols) and DPPG-polymyxin B at $R_i = 5$ (full symbols) derived from the $h_{2930}/h_{2880}$ (triangles) and $h_{2880}/h_{2845}$ (circles) peak height intensity ratios. The dashed line is the uncorrected $h_{2930}/h_{2880}$ profile.

Fig. 4 shows the temperature profiles derived from the $h_{2930}/h_{2880}$ ratio for both polymyxin bound and free DPPG. It can be seen that the phase transition temperature is lowered from 41°C to 37°C when polymyxin B is added to DPPG and that at all temperatures the overall disorder is lower for the bound lipid.

All the results shown above for the DPPG-polymyxin B complex were obtained for an incubation molar ratio of 5 ($R_i = 5$), which represents the charge saturation condition. Since for this complex no trace of unbound polymyxin or DPPG was found in the Raman spectrum of the supernatant, the real molar ratio of the white pellet studies was 5. We have also prepared a complex at $R_i = 15$ in order to determine if there is a lateral phase separation in the presence of an excess of lipid. As for the $R_i = 5$ complex, a white pellet was obtained after centrifugation and its thermal behavior was exactly as that of the $R_i = 5$ complex. In fact, the excess lipid was found in the aqueous supernatant. Therefore, the phase separation detected from our Raman results is not lateral as proposed from the fluorescence data [13], but is instead macroscopic.

*Infrared spectroscopy*

In the infrared spectra of DPPG and DPPG-polymyxin complexes, the following regions were investigated: (1) the C-H stretching mode region (2800–3000 $cm^{-1}$); (2) the carbonyl stretching mode region (1680–1780 $cm^{-1}$); (3) the

phosphate stretching mode region (1150–1300 $cm^{-1}$). These regions give information about different parts of the lipid molecule, that is the acyl chains, the interface between the hydrophobic and hydrophillic parts, and the polar head group region, respectively. Fig. 5 shows the acyl chain C-H stretching mode region for pure DPPG (solid line) and DPPG-polymyxin B complex at $R_i = 5$ (dashed line) below and above the phase transition temperature of the lipid. This spectral region is dominated by two strong bands, the methylene antisymmetric stretching mode near 2920 $cm^{-1}$, and the methylene symmetric stretching mode near 2850 $cm^{-1}$. Weaker bands due to the asymmetric and symmetric stretching modes of the terminal methyl group are also observed near 2950 and 2870 $cm^{-1}$, respectively. The two methylene bands exhibit the same behaviour as the temperature is increased: they become broader and they shift to higher frequency. Earlier studies have assigned the bandwidth increase to the augmentation of the rotational mobility of the acyl chains, and frequency shift to the introduction of *gauche* conformers in the high temperature acyl chains [31]. However, experiments made in our laboratory on hydrocarbons trapped in urea clathrates, in which case they can only adopt the trans conformation, have shown that the frequency of the methylene C-H stretching mode bands is temperature dependent even if the chain conformation remains unchanged (unpublished observations). However, since the slope of this variation is rather small (approx. 0.5 $cm^{-1}/40$ Cdeg), we assume that for two DPPG molecules at the same temperature, the value of the maximum intensity frequency can be taken as a probe for *gauche / trans* conformation.

Fig. 6 shows temperature profiles derived from the symmetric methylene stretching mode band near 2850 $cm^{-1}$. Similar results were obtained with the band due to the antisymmetric stretching mode, but with more dispersion because of overlapping vibrational modes. Curves shown on Fig. 6 for both pure and polymyxin bound DPPG are in very good agreement with the Raman results since the phase transition temperature of DPPG is also lowered from 41 °C to 37 °C and is still highly cooperative in presence of polymyxin B. These curves also demonstrate that below the phase transition temperature, polymyxin B does not affect the conformation of the acyl chains of DPPG. Above the transition temperature, even

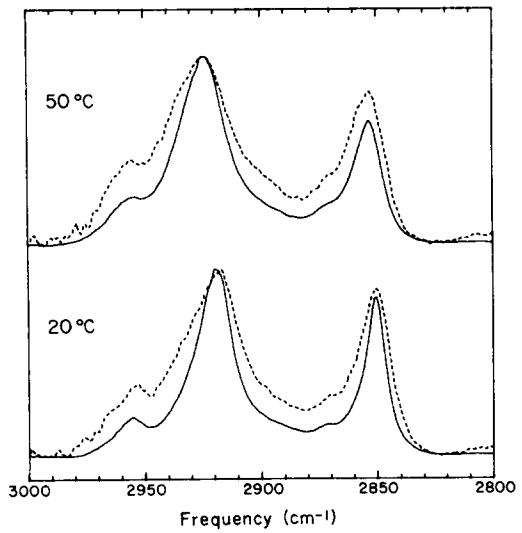


Fig. 5. Infrared spectra of DPPG (solid line) and DPPG-polymyxin B at $R_i = 5$ (dashed line) at 20 °C and 50 °C in the C-H stretching region.

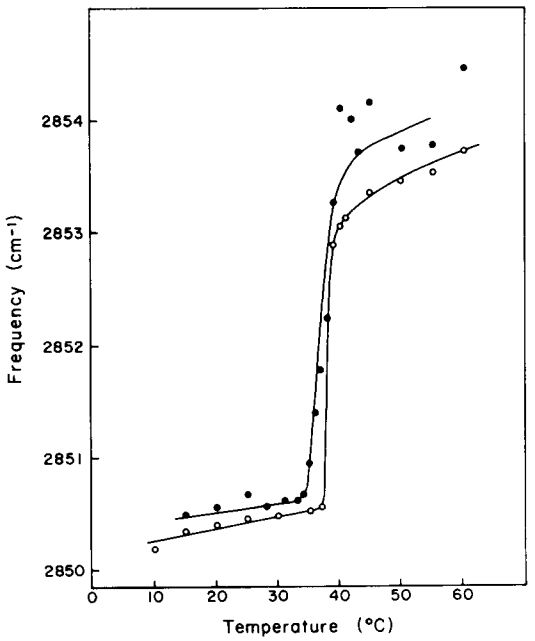


Fig. 6. Temperature profiles of DPPG (open circles) and DPPG-polymyxin B at $R_i = 5$ (full circles) derived from the frequency of the methylene symmetric stretching mode.

though there is a significant scattering of the data points, it seems that there is more conformational disorder in DPPG-polymyxin complexes, again in good agreement with the Raman results.

The second spectral region of interest in the infrared spectrum of DPPG is the carbonyl stretching mode region which ranges from 1680 to 1780 $cm^{-1}$. In order to gain valuable information from this region, spectra were first corrected for the water contribution as described above. Fig. 7A shows the results obtained for pure DPPG (solid line) and the $R_i = 5$ DPPG-polymyxin B complex (dashed line) below and above the phase transition temperature of the lipid. As can be seen, the spectra of pure and polymyxin bound DPPG are quite similar. Fig. 7B shows the same spectral region but after Fourier deconvolution with a Lorentzian line of 18 $cm^{-1}$ and a $K$ factor of 2.2 [19]. Under these conditions, two main features are clearly seen. By comparison with previous data on phospholipids, the high- and low-frequency components can be assigned to the C=O stretching vibration of carbonyl groups with *trans* or *gauche* conformation about the $C_1$-$C_2$ bond in the ester linkage, respectively [32,33]. For pure DPPG, at temperature below the gel to liquid-crystalline

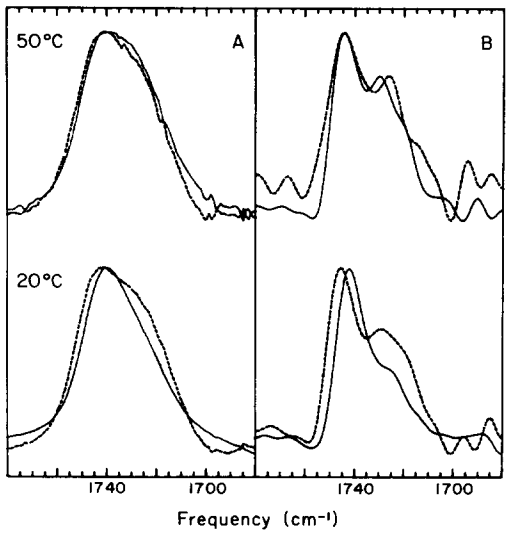


Fig. 7. Infrared spectra of DPPG (solid line) and DPPG-polymyxin B at $R_i = 5$ (dashed line) in the C=O stretching mode region at 20 °C and 50 °C. (A) Water contribution corrected spectra. (B) Fourier deconvolved water corrected spectra (Lorentzian line 18 $cm^{-1}$ wide and $K = 2.2$ [19]).

phase transition, the *trans* contribution is much more important than that of the *gauche* conformation. When polymyxin B is added, the two bands become more equivalent in intensity. Temperature elevation affects the spectrum of pure DPPG in the same way than polymyxin does in the gel phase, that is the *trans* and *gauche* contributions become more equivalent. *Gauche/trans* intensity ratio does not change with temperature for the complex.

The frequency of the carbonyl bands is also considerably affected by the presence of polymyxin B. In the gel phase at 20 °C, for example, the *trans* component at higher frequency shifts from 1742 to 1746 $cm^{-1}$ when polymyxin B is added. The *gauche* component at lower frequency seems to display a similar behaviour, but since this band is not well resolved, it is difficult to determine its frequency with accuracy. As temperature is increased, the *trans* component of the C=O band of the DPPG-polymyxin $R_i = 5$ complex is shifted to lower frequency while that of pure DPPG is shifted towards higher frequency, which is the normal behaviour for pure phospholipids. The result of these two opposite shifts is that at 50 °C the frequency of the trans band of the bound lipid coincides with that of pure DPPG. Such a behaviour as that of the C=O stretching mode of polymyxin bound DPPG, a shift to lower frequency upon temperature elevation, has not been reported yet in the literature.

The last region of the infrared spectra that was analysed was that from 1150 to 1300 $cm^{-1}$, where bands due to phosphate vibrations occur. Fig. 8 shows this spectral region for pure (solid line) and bound (dashed line) DPPG at 20 °C. The main feature in this region is the $O{=}P{-}O^-$ antisymmetric stretching mode band centered at about 1210 $cm^{-1}$. The weaker bands in these spectra are due to the acyl chains methylenes wagging progression [34] and they superimpose with the $PO_2^-$ band. As seen in Fig. 8, the introduction of polymyxin B in a DPPG dispersion at 20 °C has practically no effect on the $PO_2^-$ antisymmetric stretching band. This is a surprising result since we know that there is a strong electrostatic interaction between DPPG and polymyxin B, and that this interaction should occur at the level of the $PO_2^-$ group, since this part of the lipid molecule is the only one bearing a

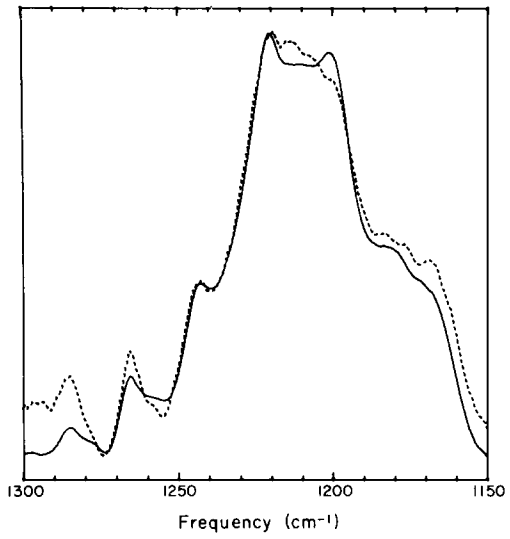


Fig. 8. Infrared spectra of DPPG (solid line) and DPPG-polymyxin B at $R_i = 5$ (dashed line) at 20 °C in the phosphate stretching region.

negative charge. Therefore, it seems that the binding of polymyxin B to DPPG does not change the double bond character of the $PO_2^-$ groups.

## Discussion

As expected from the number of positive charges borne by the polymyxin B at physiological pH, the antibiotic binds five phosphatidylglycerol molecules [5,7]. In the presence of excess of phosphatidylglycerol at $R_i = 15$, Theretz et al. [13] have reported two transitions from fluorescence polarization measurements that were assigned to free and bound DPPG molecules, therefore suggesting lateral phase separation as observed in the case of complexes of polymyxin B and phosphatidic acids [9]. Our results at $R_i = 15$ demonstrate clearly that there is only one phase transition and that no lateral phase separation occurs. Instead, the free lipid was found in the supernatant phase, indicating a macroscopic rather than a lateral phase separation. The apparent discrepancy between our results and those of Theretz et al. [13] is most likely due to the fact that during the fluorescence polarization measurements, samples were continuously agitated which prevented precipitation but also made the distinction between lateral and macroscopic phase separation impossible. To check this hypothesis, we have recorded a DPH fluorescence polarization curve of a DPPG-polymyxin B complex at $R_i = 15$ without agitating the sample, and only one transition was detected, corresponding to the gel to liquid-crystalline phase transition of pure DPPG (unpublished results). Therefore, polymyxin B does not seem to induce the formation of domains of peptide bound DPPG in a matrix of a bilayer of unbound DPPG, as opposed to other peptides like poly(L-lysine) [26] and melittin (Lafleur, M. and Pézolet, M., unpublished results.).

Both Raman and infrared results demonstrate clearly that the phase transition temperature of DPPG is decreased from 41 °C to 37 °C when the phospholipid is bound to polymyxin B, which is in good agreement with the fluorescence results of Theretz et al. [13] and Sixl and Galla [7]. However, we have never detected the double transition reported from differential scanning calorimetry experiments by Theretz et al. [13] and by Bogs and Rangaraj [14]. The two transitions, separated by about two degrees and occurring near the phase transition temperature of the pure lipid, were attributed to the transition from an interdigitated gel phase of DPPG to an uninterdigitated gel phase, and then to the liquid-crystalline state. Since the biphasic transition for DPPC-polymyxin B complexes has been detected only by differential scanning calorimetry and not by fluorescence and vibrational spectroscopy, for which the thermal equilibration period is rather long and allows the system to reach the thermodynamically stable state, it is likely that the transition detected by calorimetry are associated with the formation of a metastable state. A similar behaviour has been reported for mixed-chain phosphatidylcholines with chain lengths differing by at least four carbon atoms [35], and was associated with the formation of a kinetically favoured metastable phase.

The temperature profiles derived from the Raman $h_{1090}/h_{1125}$ intensity ratio and from the frequency of the infrared activity methylene C-H stretching modes show that polymyxin B does not affect significantly the conformation of the acyl chains of DPPG in the gel state. Since the presence of the hydrophobic part of proteins and polypeptides within the hydrophobic core of lipid membranes generally given rise to the appearance

of more *gauche* conformers along the acyl chains of phospholipids in the gel phase [36–39], it seems that the hydrophobic tail of polymyxin B does not penetrate the hydrophobic matrix of the bilayer at temperature below the phase transition of the DPPG-polymyxin complex. For the gel phase, the Raman $h_{2880}/h_{2845}$ intensity ratio shows that the intermolecular vibrational coupling between the acyl chains of DPPG is more important in the bound than in the pure lipid. A similar effect has been observed for the polylysine-DPPG system [40] and has been associated to the decrease of the tilt angle of the acyl chains upon neutralization of the polar headgroup charges of the lipid by the polypeptide. Interdigitation of the lipid acyl chains can also lead to high values of the $h_{2880}/h_{2845}$ intensity ratio [27].

Infrared data on the carbonyl stretching mode region also provide valuable information on the structure of DPPG-polymyxin complexes below the phase transition temperature. As seen from the relative intensity of the two carbonyl bands after Fourier deconvolution (Fig. 7B), the binding of polymyxin B to DPPG modifies the conformation of the glycerol backbone of the lipid in such a way that more gauche conformers about the $C_1$-$C_2$ bond in the ester linkage are present. In fact, the spectrum of the $R_i = 5$ complex at 20 °C is very similar to that of pure DPPG in the liquid-crystalline phase. This behaviour is completely different from that observed for complexes of DPPG with either $Ca^{2+}$ ions or poly(L-lysine). In the first case, the C=O band is split in three sharp components (Besner, D. and Pézolet, M., unpublished results) due to a crystalline field effect in the cochleate structure adopted by charged lipids in the presence of $Ca^{2+}$ ions [43]. A similar splitting has also been observed for phosphatidylserine-calcium complexes [44]. On the other hand, infrared spectra of complexes of poly(L-lysine) and DPPG display essentially only the high frequency C=O component suggesting that in the presence of this polypeptide the two C=O groups of DPPG are more equivalent than those for the pure lipid. The effect of polymyxin B on DPPG is exactly the opposite. The frequency of the carbonyl bands in the DPPG-polymyxin system is also peculiar. The high-frequency component appears at 1746 $cm^{-1}$ instead of 1742 $cm^{-1}$ for the pure lipid. Such a high value for a carbonyl band of a phospholipid has not yet been reported in the litterature.

All the above spectral results lead to the conclusion that the structure adopted by DPPG-polymyxin B complexes in the gel state is different from that of other lipidic systems and might be associated with interdigitation of the lipid acyl chains as suggested by Ranck and Tocanne [15]. In order to confirm this conclusion we have determined by small-angle X-ray diffraction that the lamellar repeat distance decreases from 5.85 nm for pure DPPG to 4.49 nm for a $R_i = 5$ DPPG-polymyxin B complex, while this parameter remains at about the same as that of pure DPPG in the case of the non-interdigitated DPPG-polylysine system. Therefore, we believe that the acyl chains are interdigitated in DPPG-polymyxin B complexes below the gel to liquid-crystalline transition, and that this structure is responsible for the high intermolecular vibrational coupling as revealed by the Raman C-H stretching mode region. In addition, the increase of the spacing of the ester groups and the resulting change of polarity of their environment due to interdigitation probably account for the change in shape and frequency of the infrared C=O bands. It is also very likely that the macroscopic phase separation is due to interdigitation since this domains of interdigitated lipid would be very unstable in thicker bilayers of unperturbed lipids.

In the liquid-crystalline phase, both the Raman $h_{1090}/h_{1125}$ intensity ratio and the frequency of the infrared active C-H methylene symmetric stretching mode show that polymyxin B induces the formation of more *gauche* conformers along the lipid acyl chains. This suggests that the hydrophobic tail of polymyxin B penetrates the hydrophobic matrix of the lipid bilayer in the liquid-crystalline state, in contrast to the behaviour of the antibiotic in the gel phase. On the other hand, the Raman $h_{2880}/h_{2845}$ intensity ratio reveals that the intermolecular vibrational coupling of the acyl chains of DPPG in the liquid-crystalline phase remains high in the presence of polymyxin B. The explanations proposed for a higher vibrational coupling in the gel phase do not, however, prevail in the liquid-crystalline phase since neither interdigitation nor the presence of a tilt angle in the acyl chains exist anymore in this phase [15,41].

However, it has recently been shown by NMR spectroscopy [42] that the motional rate of the glycerol headgroup of DMPG in the liquid-crystalline state is restricted by polymyxin B. Therefore, it is very likely that the motional rate of the whole DPPG molecule is restricted by polymyxin, thus allowing more intermolecular vibrational coupling.

Increasing the temperature above the phase transition of DPPG-polymyxin complex does not seem to affect significantly the conformation of the glycerol backbone of DPPG since the relative intensity of the carbonyl bands remains constant. However, the doublet is shifted to lower frequency and above the phase transition of the lipid it is almost identical to the carbonyl band of the pure lipid. Therefore, even though the hydrophobic tail of polymyxin appears to penetrate the bilayer at high temperature, it does not change the conformation of the ester linkages. This result is in agreement with the NMR results [42] showing that the time average structure of the headgroup of DMPG is not modified by polymyxin B.

From all the results presented above, the increase of the intermolecular interaction and the decrease of the phase transition temperature of DPPG upon addition of polymyxin B may appear to be contradictory. One would expect that when the positive charges of polymyxin neutralize the negative charges of DPPG molecules the molecular packing becomes tighter, therefore shifting the phase transition temperature to higher temperature. This is indeed what has been observed in the case of DPPG-polylysine interactions [40]. We propose the following model to explain this behaviour of the DPPG-polymyxin B complex. In the gel phase, under physiological pH conditions, the DPPG molecules bear one negative charge and because of electrostatic repulsion, their headgroups occupy a larger volume and their chains are tilted by about 30° relative to the bilayer normal to maximize the hydrophobic interactions [45]. Upon interaction of five DPPG molecules with one polymyxin B molecule, there is a neutralization of the charges of the lipid and a tightening of the lipid assembly due to the loss of the tilt angle of the acyl chains and to their interdigitation. Because of this tight assembly, the hydrophobic tail of polymyxin B cannot penetrate into the hydrophobic part of the lipid bilayer and the tail is then in an unstable state. This instability of the polymyxin tail triggers the phase transition of DPPG at a slightly lower temperature so that the hydrophobic chain of polymyxin B can penetrate the hydrophobic matrix of the bilayer. The fact that the cooperativity of the phase transition of bound DPPG is as high as that of the pure lipid also supports this model.

In conclusion, our results show that each polymyxin B molecule binds five DPPG molecules at physiological pH and induces a macroscopic phase separation of the complex from unperturbed bilayers. Below the phase transition of DPPG/polymyxin B bilayers, results obtained by Raman and infrared spectroscopies and by small angle X-ray diffraction suggest that the acyl chains of DPPG are interdigitated and that the hydrophobic tail of the antibiotic does not penetrate this tight assembly. However, polymyxin shifts to 37°C the transition of DPPG which remains highly cooperative. Above the phase transition of the lipid bilayer, the conformation of the acyl chains of DPPG is more disordered due to the penetration of the polymyxin chain but the structure of the glycerol backbone of DPPG does not seem to be affected. However, the motional rate of the lipid is more restricted.

## Acknowledgements

We are indebted to G. Laroche for performing the X-ray diffraction measurements. This work was supported by grants from the Natural Science and Engineering Research Council of Canada and from the Fonds pour la Formation de Chercheurs et Aide à la Recherche of the Ministère de l'Education of the Province of Québec.

## References

1 Storm, D.R., Rosenthal, R.S. and Swanson, P.E. (1977) Annu. Rev. Biochem. 46, 723–763
2 Imai, M., Inoue, K. and Nojima, S. (1975) Biochim. Biophys. Acta 375, 130–137
3 Hsu Chen, C.C. and Feingold, D.S. (1973) Biochemistry 12, 2105–2111
4 Feingold, D.S., Hsu Chen, C.C. and Sud, I.J. (1974) Ann. N.Y. Acad. Sci. 235, 480–492
5 El Mashak, E.M. and Tocanne, J.F. (1980) Biochim. Biophys. Acta 596, 165–179

6 Mushayakarara, E. and Levin, I.W. (1984) Biochim. Biophys. Acta 769, 585–595
7 Sixl, F. and Galla, H.J. (1981) Biochim. Biophys. Acta 643, 626–635
8 Hartmann, W., Galla, H.J. and Sackmann, E. (1978) Biochim. Biophys. Acta 510, 124–139
9 Sixl, F. and Galla, H.J. (1979) Biochim. Biophys. Acta 557, 320–330
10 Sixl, F. and Galla, H.J. (1980) Biochim. Biophys. Res. Commun. 94, 319–323
11 Galla, H.J. and Trudell, J.R. (1980) Biochim. Biophys. Acta 602, 522–530
12 Sixl, F. and Galla, H.J. (1982) Biochim. Biophys. Acta, 693, 466–478
13 Theretz, A., Ranck, J.L and Tocanne, J.F. (1983) Biochim. Biophys. Acta 732, 499–508
14 Boggs, J.M. and Rangaraj, G. (1985) Biochim. Biophys. Acta 816, 221–233
15 Ranck, J.L. and Tocanne, J.F. (1982) FEBS Lett. 143, 175–178
16 Chapman, D. and Hayward, J.A. (1985) Biochem. J. 228, 281–295
17 Tu, A.T. (1982) in Raman Spectroscopy in Biology: Principles and Applications, pp. 187–233, John Wiley and Sons, New York
18 Savoie, R., Boulé, B., Genest, G. and Pézolet, M. (1979) Can. J. Spectrosc. 24, 112–117
19 Kauppinen, J.K., Moffat, D.J., Mantsch, H.H. Cameron, D.G. (1981) Anal. Chem. 53, 1454–1457
20 Lippert, J.L. and Peticolas, W.L. (1971) Proc. Natl. Acad. Sci. USA 68, 1572–1576
21 Spiker, R.C., Jr. and Levin, I.W. (1975) Biochim. Biophys. Acta 388, 361–373
22 Yellin, N. and Levin, I.W. (1977) Biochemistry 14, 4870–4876
23 Gaber, B.P. and Peticolas, W.L. (1977) Biochim. Biophys. Acta 465, 260–274
24 Snyder, R.G., Scherer, J.R. and Gaber, B.P. (1980) Biochim. Biophys. Acta 601, 47–53
25 Kouaouci, R., Silvius, J.R., Graham, I. and Pézolet, M. (1985) Biochemistry 24, 7132–7140
26 Carrier, D. and Pézolet, M. (1986) Biochemistry 25, 4167–4174
27 O'Leary, T.J. and Levin, I.W. (1984) Biochim. Biophys. Acta 776, 185–189
28 Hill, I.R. and Levin, I.W. (1979) J. Chem. Phys. 70, 842–851
29 Dasseux, J.L., Faucon, J.F., Lafleur, M., Pézolet, M. and Dufourcq, J. (1984) Biochim. Biophys. Acta 775, 37–50
30 Bunow, M.R. and Levin, I.W. (1977) Biochim. Biophys. Acta 487, 388–394
31 Umemura, J., Cameron, D.G. and Mantsch, H.H. (1980) Biochim. Biophys. Acta 602, 32–34
32 Bicknell-Brown, E., Brown, K.G. and Person, W.B. (1980) J. Am. Chem. Soc. 102, 5486–5491
33 Levin, I.W., Mushayakarara, E. and Bittman, R. (1982) J. Raman Spectrosc. 13, 231–234
34 Casal, H.L. and Mantsch, H.H. (1984) Biochim. Biophys. Acta 779, 381–401
35 Mason, J.T., Huang, C.H. and Biltonen, R.L. (1981) Biochemistry 20, 6086–6092
36 Dunker, A.K., Williams, R.W., Gaber, B.P. and Peticolas, W.L. (1979) Biochim. Biophys. Acta 553, 351–357
37 Curatolo, W., Verma, S.P., Sakura, J.D., Small, D.M., Shipley, G.G. and Wallach, D.F.H. (1978) Biochemistry 17, 1802–1807
38 Susi, H., Sampugna, J., Hampson, J.W. and Ard, J.S. (1979) Biochemistry 18, 297–301
39 Taraschi, T. and Mendelsohn, R. (1979) J. Am. Chem. Soc. 101, 1050–1052
40 Carrier, D. and Pézolet, M. (1984) Biophys. J. 46, 497–506
41 Ranck, J.L., Keira, T. and Luzatti, V. (1977) Biochim. Biophys. Acta 488, 423–441
42 Sixl, F. and Watts, A. (1985) Biochemistry 24, 7906–7910
43 Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. (1976) Biochim. Biophys. Acta 448, 365–383
44 Dluhy, R.A., Cameron, D.G., Mantsch, H.H. and Mendelsohn, R. (1983) Biochemistry 22, 6318–6325
45 Watts, A., Harlos, K. and Marsh, D. (1981) Biochim. Biophys. Acta 645, 91–96