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# Comparison of the Effects of Amikacin and Kanamycins A and B on Dimyristoylphosphatidylglycerol Bilayers

## AN INFRARED SPECTROSCOPIC INVESTIGATION

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**ABSTRACT.** Aminoglycoside antibiotics are very effective against severe Gram-negative infections, but their clinical use is associated with nephrotoxic side-effects. The cascade of events leading to acute renal failure involves an impairment of lysosomal phospholipase activity, which is thought to result from the direct interaction of the drugs with the head group of negative phospholipids. Fourier transform infrared spectroscopy was used to study the effects of three aminoglycosides from the kanamycin family (amikacin and kanamycins A and B) on dimyristoylphosphatidylglycerol (DMPG) bilayers at lysosomal pH. The results obtained were consistent with a tightening of the lipidic network caused by the neutralization of the negative head groups of DMPG by the positive charges of the aminoglycosides. These antibiotics induced an increase of the transition temperature of DMPG, a decrease of both the frequency and relative intensity of the hydrogen-bonded carbonyl component, and a decrease of the phosphate antisymmetric band frequency. Kanamycin B, which is known to be the most nephrotoxic drug of the three, exhibited the greatest effects on the transition temperature and on the carbonyl stretching band. A comparison of the nature and extent of the spectral changes led us to conclude that amikacin lies flat on the bilayer surface, whereas kanamycin B is located between the lipidic head groups and quite close to some of the carbonyl groups. Finally, a possible correlation between the importance of bilayers perturbation and the respective inhibitory potency against phospholipases was examined.  BIOCHEM PHARMACOL 53;3:401–408, 1997.

**KEY WORDS.** amikacin; kanamycin A; kanamycin B; dimyristoylphosphatidylglycerol; infrared spectroscopy

Aminoglycoside antibiotics show very good efficacy against life-threatening Gram-negative infections but, unfortunately, they can also lead to acute renal failure. Despite the continual introduction of new drugs, this class of antibiotics remains widely prescribed because there is still no satisfactory alternative. The understanding of the molecular mechanism responsible for their nephrotoxicity could greatly help in the design of less nephrotoxic structures. The aminoglycosides can only be eliminated by kidney glomerular filtration, but they tend to accumulate in high concentrations in the proximal tubule cells during the subsequent reabsorption step. Pastoriza-Munoz *et al.* [1] used the microinjection technique to demonstrate that [$^3$H]gentamicin was absorbed along the proximal convoluted tubule and loop of Henle of superficial nephrons. Autoradiographic studies showed that gentamicin is reabsorbed from the glomerular filtrate by pinocytosis and is stored in the lysosomes of the proximal tubule cells [2]. The administration of these antibiotics to living organisms was shown previously to result in a marked increase in the total volume of lysosomes, which accumulated dense lamellar structures called myeloid bodies consisting of concentrically arranged, densely packed membranes [3]. This event suggests an impairment of the phospholipase activity. An excessive growth of the myeloid bodies in lysosomes follows and results in their swelling and bursting, which could be responsible for cell death due to the release of large amounts of the aminoglycoside, lysosomal enzymes, and phospholipids into the cytosol [3–5].

Jain *et al.* [6] demonstrated that the activity of these phospholipases depends on the membrane fluidity and on the net negative charge on the surface of the bilayers. Under conditions that mimic the lysosomal environment, the polycationic drugs bind to the negatively charged phospholipid bilayers through electrostatic interactions, since ionic strength and pH both effect the binding [3]. It is hypothesized that the antibiotics impair indirectly the activity of the enzyme by neutralizing the negative charge of phospholipids and decreasing membrane fluidity. The physical effects of three aminoglycosides on dimyristoylphosphatidyl-

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† *Abbreviations:* DMPG, dimyristoylphosphatidylglycerol; FTIR, Fourier transform infrared spectroscopy; PC, phosphatidylcholine; PI, phosphatidylinositol; $PLA_2$, phospholipase $A_2$; and PS, phosphatidylserine.

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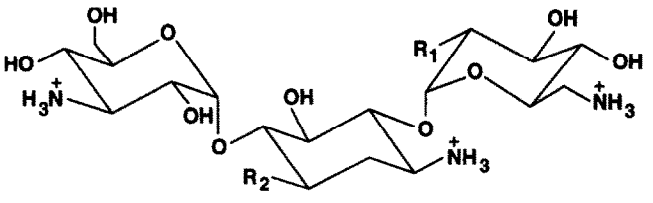
glycerol bilayers at pH 5.4, which is the pH found in the lysosomes [7], are examined in the present study. The fluidity of the liposomes in the absence and in the presence of these drugs was investigated using FTIR† spectroscopy.

The antibiotics under study are amikacin and kanamycins A and B, all of which belong to the kanamycin family (Fig. 1). Kanamycin A contains a deoxystreptamine unit that is linked in the α configuration to 6-amino-6-deoxy-D-glucose at the 4-position and to 3-amino-3-deoxy-D-glucose at the 6-position [8]. Kanamycin B is a natural derivative of kanamycin A, and it bears one additional charge. The structures of kanamycin A and amikacin, a semisynthetic derivative of kanamycin, are the same except for one group (Fig. 1). The changes induced by the aminoglycoside antibiotics in the infrared spectra of the lipid bilayers, especially in the frequency windows relevant to their acyl chains and head group moieties, give an insight into the mechanism of toxicity of these drugs.

## MATERIALS AND METHODS

Amikacin, the sulfate salts of kanamycins A and B, and the sodium salt of dimyristoyl (C14:0) L-α phosphatidyl-DL-glycerol were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deuterated water was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

The lipidic dispersions (5%, w/v) for infrared measurements were prepared by mixing the appropriate amounts of lipid and solvent ($H_2O$ or $D_2O$). Experiments were performed at pH 5.4, the pH found in the lysosomes where aminoglycosides accumulate in kidney cells [1, 7]. The pH was adjusted by adding minimal amounts of NaOH, HCl or deuterated NaOH, HCl. At least three freeze-thaw cycles were done to ensure proper organization of the bilayers (liposomes), and the pH was checked again. The amount of a 5% (w/v) aminoglycoside solution (pH 5.4) required to give a DMPG:aminoglycoside molar ratio of 4:1 was added to an aliquot of the lipid dispersion, and the mixture was vortexed. After checking the pH again, the sample was submitted to another set of freeze–thaw cycles. The sample was then centrifuged briefly to concentrate the visible aggregates in the bottom of the tube, and the pellet was used for measurements. The sample was placed between two calcium fluoride windows separated by a 6-μm spacer. A thermostat-equipped cell mount and a variable temperature water bath were used to heat the sample from 5 to 50°. The spectra were recorded on a Digilab FTS-40A with a spectral resolution of 2 $cm^{-1}$. Two hundred fifty-six interferograms were co-added for each spectrum. The instrument was purged continuously with dry air to eliminate spectral contributions from atmospheric water vapor. The data were processed with homemade software (National Research Council, Canada). Resolution enhancement of overlapping bands was done using Fourier self-deconvolution [9], whereas frequencies were determined with the aid of Fourier derivation [10].



| | $R_1$ | $R_2$ |
|---|---|---|
| Amikacin | OH | NH-C(=O)-CHOH-$CH_2CH_2NH_3^+$ |
| Kanamycin A | OH | $NH_3^+$ |
| Kanamycin B | $NH_3^+$ | $NH_3^+$ |

**FIG. 1. Chemical structures of the aminoglycosides amikacin and kanamycins A and B.**

## RESULTS

Infrared spectroscopy can efficiently detect interactions localized at individual functional groups [11], and it was employed here to study the interactions of three members of the kanamycin family with DMPG bilayers. Four characteristic bands of the lipid spectrum are particularly useful: the carbonyl stretching band, the methylene stretching and scissoring bands, and the phosphate antisymmetric stretching band, all of which occur in spectral regions with no significant aminoglycoside contributions.

The physical state of liposomal lipidic bilayers depends on temperature. The gel state, found at low temperature, is a state in which there is little movement within the hydrocarbon chains. Raman spectroscopic studies indicated that the rotations about the carbon—carbon bonds of the hydrocarbon chains become more difficult as the temperature is lowered [12]. Therefore, the hydrocarbon skeleton tends to adopt an *all trans* conformation in the gel state. When the temperature is increased, the bilayers go from a gel state to a liquid-crystal state. Above the transition temperature, the hydrocarbon chains contain a large proportion of gauche conformers and exhibit much higher rates of intra- and intermolecular motions.

### *Effect of the Aminoglycosides on the Fluidity of the Liposomes*

The effects of amikacin and kanamycins A and B on the thermotropic properties of negatively charged DMPG liposomes were studied at pH 5.4, the lysosomal pH [7]. The temperature was raised from 5° to 50°, and the band corresponding to the symmetric stretching mode of the methylene group, found at a frequency of about 2850 $cm^{-1}$, was studied. This vibration is sensitive to conformational changes in the acyl chains of lipid bilayers and can be used to probe membrane fluidity. The frequency of the methylene symmetric stretching band (2850 $cm^{-1}$) is known to

increase with the number of gauche conformers in the acyl chains [13, 14]. Since the lipidic gel-to-fluid phase transition involves an important increase in the proportion of gauche bonds within the hydrocarbon chains, a plot of the frequency of this mode against temperature will contain a discontinuity at the transition temperature (Fig. 2). The transition temperature of DMPG alone was 25.5° (Fig. 2, open circles), and it rose to 26.7° (filled circles), 26.4° (triangles), and 26.2° (squares), respectively, when kanamycin B, kanamycin A, and amikacin were added in a molar ratio of four DMPG to one antibiotic molecule. Moreover, all three antibiotics induced a decrease of the frequency of the $CH_2$ symmetric stretching band in both the gel and liquid crystalline phases (Fig. 2). As indicated above, kanamycin B induces the largest shift of the transition temperature. The differences observed between the three antibiotics were small but reproducible. It should also be noted that the transitions remained cooperative in the presence of the aminoglycosides, which means that the drug distribution was homogeneous in the liposomes.

The effects of our three aminoglycosides on DMPG methylene scissoring vibrations were also examined. This band must be studied in $H_2O$ instead of $D_2O$ to avoid H-O-D vibrations that would appear in this spectral region. The scissoring bands of DMPG in the gel (dashed line) and

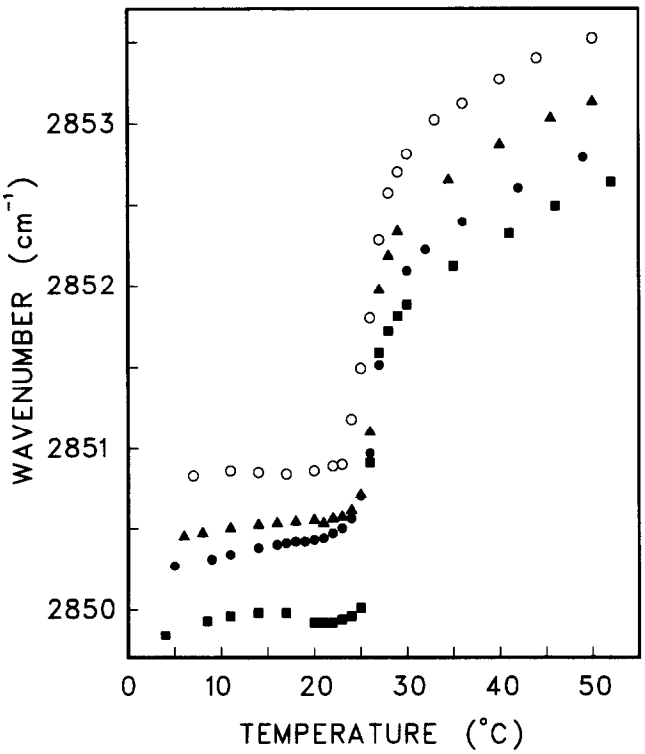



**FIG. 2. Temperature dependence of the symmetric C—H stretching vibration of the methylene groups of DMPG in aqueous dispersions at pH 5.4, in the absence (open circles) and in the presence of kanamycin B (filled circles), kanamycin A (triangles), and amikacin (squares) in a molar ratio of 4 DMPG to 1 aminoglycoside. The frequencies were obtained after Fourier derivation of the original FTIR spectra with a power of 3 and a breakpoint of 0.3.**

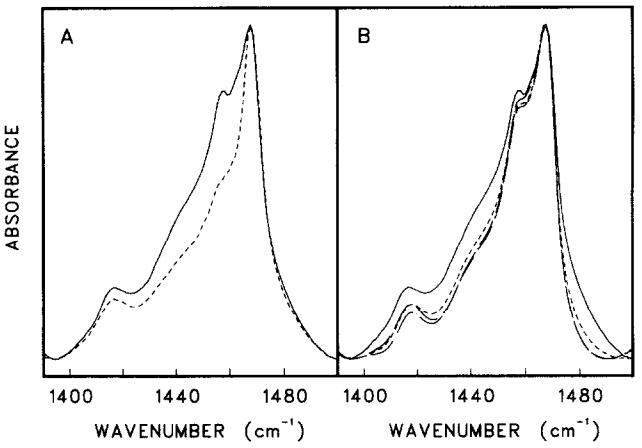



**FIG. 3. Methylene scissoring region of the infrared spectra of aqueous dispersions of DMPG at pH 5.4. Panel A shows DMPG alone in the gel state at 20° (small dashes) and in the liquid–crystal phase at 40° (solid line). Panel B: DMPG in the absence (solid line) and in the presence of kanamycin B (small dashes), kanamycin A (long dashes), and amikacin (dot-long dash) in the liquid-crystal state at 28°. The samples were prepared in $H_2O$.**

liquid–crystal (solid line) states are given in Fig. 3A. This complex band is dominated by contributions from the scissoring vibration of the methylene groups of the acyl chains at 1468 $cm^{-1}$ [15]. The deformation of the $CH_2$ group adjacent to the carbonyl function gives a small band at ~1417 $cm^{-1}$ [16], and the scissoring mode of the gauche methylene groups produces the shoulder at ~1457 $cm^{-1}$ [17], which becomes more intense in the fluid phase (solid line). The shape of the scissoring band of phospholipids in the gel phase depends on the packing symmetry of the hydrocarbon chains: a single band is observed at 1468 $cm^{-1}$ for the most common hexagonal subcell (and also for the triclinic subcell) but that band splits into a doublet at 1462 and 1473 $cm^{-1}$ due to interchain coupling in orthorhombic subcells [18]. The scissoring band of DMPG in the liquid–crystal state both in the absence (solid line) and in the presence of the aminoglycosides are depicted in Fig. 3B. To ensure that the samples were in the liquid-crystal state, the frequency of the methylene C—H stretching bands was checked, and all the samples were found to be in the liquid-crystal state. As observed in Fig. 3B, the addition of aminoglycosides to DMPG sharpened the methylene scissoring band.

### *Carbonyl Stretching Vibrations of DMPG*

The carbonyl stretching vibrations provided additional information about the bilayer state. The carbonyl stretching region of DMPG consisted of a broad band (Fig. 4, A and B, solid line), which on Fourier self-deconvolution [19] was found to contain two overlapping components (Fig. 4, C and D, solid line). The low frequency component is attributed to the stretching vibration of carbonyl groups involved in hydrogen bonds, whereas the high frequency component

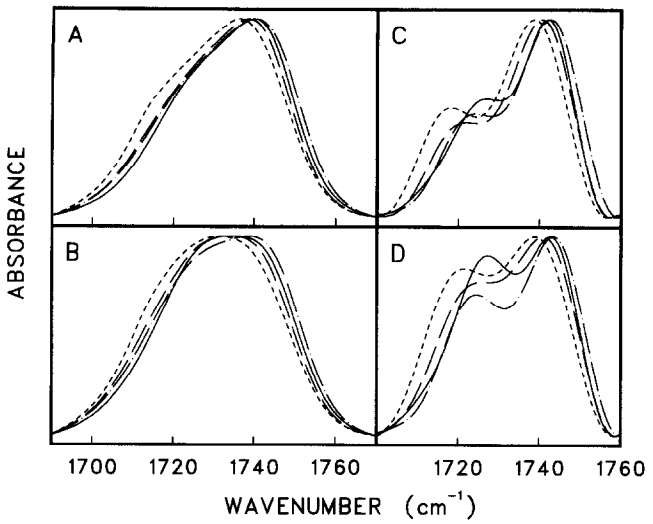



**FIG. 4. Carbonyl stretching region of the infrared spectra of dispersions of DMPG in the absence (solid line) and in the presence of kanamycin B (small dashes), kanamycin A (long dashes), and amikacin (dot-long dash), with the lipid in the gel state at 20° (panel A) or in the liquid–crystalline state at 40° (panel B). The samples were prepared in $^2H_2O$, with a final p$^2$H of 5.4. The spectra in C and D were obtained from A and B, respectively, after resolution enhancement by Fourier self-deconvolution with a bandwidth of 17 and a resolution enhancement factor of 1.5.**

is due to free C=O groups [20]. When DMPG underwent its thermotropic phase transition from gel to liquid–crystal, the relative intensity of the hydrogen-bonded carbonyl component increased (compare the solid line in panels C and D of Fig. 4). As mentioned above, the hydrocarbon chains become disordered at high temperature, causing an expansion of the lipidic bilayer, which facilitates the penetration of water to the interface and thus favors hydrogen bonding to the ester groups. Besides that intensity change, the modification in the shape of the overall band was due partly to an increase of the frequency of the hydrogen-bonded carbonyl component with temperature. As seen on Fig. 5A, the frequency of the hydrogen-bonded carbonyl band for the lipid alone increased by approximately 4 cm$^{-1}$ over the 40° temperature range considered. Thus, at high temperature, the hydrogen bond between the lipid C=O group and the hydrogen of the solvent, in this case deuterated water, was weaker than at low temperature.

When the bilayer was in the gel state, the aminoglycosides induced a slight decrease of the relative intensity of the hydrogen bonded carbonyl stretching band, which was observed more easily after deconvolution (Fig. 4C). This means that the addition of kanamycin A or B or amikacin reduces the availability of hydrogen bond donors in the bilayer interface region. Gurnani *et al.* [21] also observed this feature when gentamicin was added to PI liposomes in a molar ratio of 2 PI to 1 gentamin. The drugs under study also caused a downward shift of the low frequency component of the carbonyl band; this decrease in frequency of about 1–5 cm$^{-1}$ can be seen by comparing the profiles in Fig. 5B to that in Fig. 5A. The lower frequency of the stretching band of the hydrogen-bonded carbonyl groups, compared with DMPG alone, indicated the formation of stronger hydrogen bonds and suggested a greater cohesion of the molecules in the bilayer. The aminoglycosides also caused a decrease of the relative intensity of the carbonyl hydrogen-bonded component of DMPG in the liquid–crystal phase (Fig. 4D). As for the pure lipid, an increase of the frequency of the hydrogen-bonded carbonyl band upon going to the liquid-crystal state was observed in the presence of aminoglycosides, but that frequency remained lower than that of DMPG alone at the same temperature. Kanamycin B exhibited the largest lowering of the frequency of the two components of the carbonyl stretching band, both in the gel and in the liquid–crystal state.

### *Phosphate Stretching Vibrations of DMPG*

Finally, the stretching vibrations of the lipid phosphate group in the liquid–crystal state were studied. Infrared bands characteristic of the phosphate occur between 1000 and 1300 cm$^{-1}$. The phosphate region must be studied in

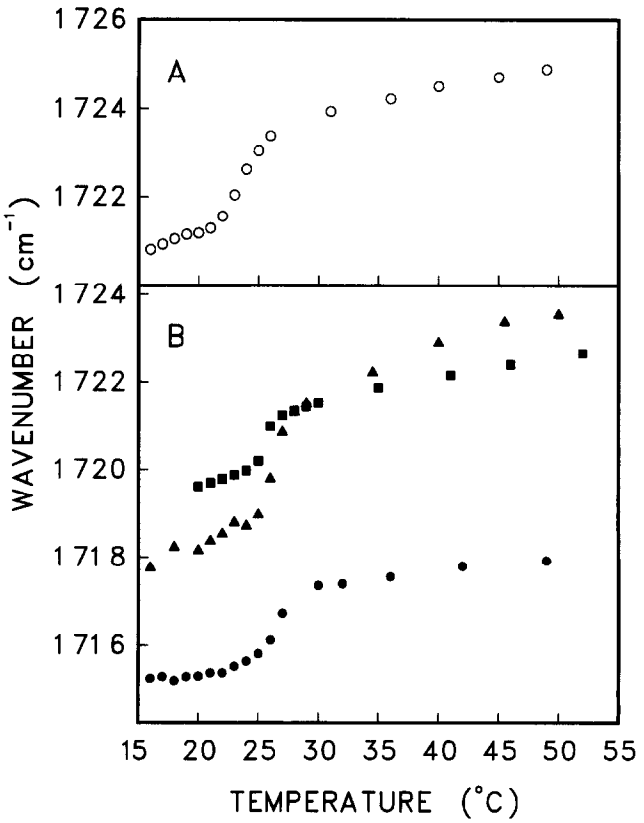



**FIG. 5. Temperature dependence of the frequency of the low frequency component of the carbonyl stretching vibration of DMPG alone (panel A) or in the presence of kanamycin B (panel B, filled circles), kanamycin A (panel B, triangles), and amikacin (panel B, squares). The samples were prepared in $^2H_2O$, with a final p$^2$H of 5.4. The frequencies were obtained after Fourier derivation of the original FTIR spectra with a power of 3 and a breakpoint of 0.2.**

$H_2O$ instead of $D_2O$ to avoid interference from the bending mode of this latter in the 1200 $cm^{-1}$ region. Figure 6 shows the spectra of a DMPG dispersion in the gel state (dashed line) (panel A) and in the liquid–crystalline phase (solid line) (panels A and B). The complex profile of the infrared bands in this region is attributable to the superposition of the progressional bands from $CH_2$ twisting and wagging modes [22] onto the antisymmetrical phosphate stretching region [13]. The phosphate band emerged more clearly in the fluid phase. The frequency of the phosphate antisymmetric stretching vibration varies between 1200 and 1270 $cm^{-1}$, depending on the hydrogen bonding strength [13]. For DMPG, it was observed at 1216 $cm^{-1}$ (Fig. 6B, solid line). The incorporation of aminoglycosides into liposomes resulted in a decrease of the frequency of the maximum absorbance of that band by 7–8 $cm^{-1}$ (Fig. 6B, dashed lines), suggesting the formation of stronger hydrogen bonds.

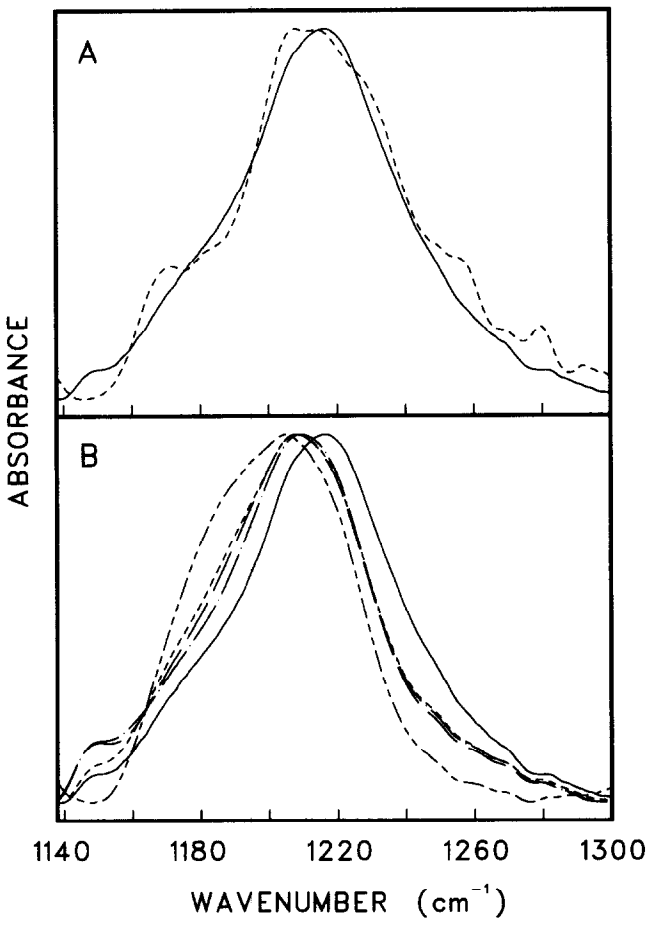



**FIG. 6. Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of DMPG at pH 5.4 in the gel state (panel A, small dashes) and in the liquid–crystal phase (panels A and B, solid line). Panel B: DMPG in the liquid-crystalline phase, pH 5.4, and in the presence of kanamycin B (small dashes), kanamycin A (long dashes), and amikacin (dot-long dash), with a molar ratio of 4 DMPG to 1 aminoglycoside. The spectrum of a dispersion of DMPG at pH 2.0 and in the fluid state is also added for comparison (panel B, dot-dot-long dash). The samples were prepared in $H_2O$.**

## DISCUSSION

Thermotropic studies using FTIR were performed to see the effects of the binding of aminoglycosides to DMPG bilayers at lysosomal pH. DMPG is a good candidate for this work: it bears a net negative charge and shows a steep transition upon going from the gel to the fluid state. Although PI is believed to be the actual receptor of aminoglycosides in kidney membranes, it was not appropriate for the present study because it could be obtained only as a mixture of molecules with different acyl chains. This heterogeneity abolishes the cooperativity of the gel-to-fluid transition. We felt that dimyristoylphosphatidylglycerol was an excellent alternative since its head group has been shown to be electrostatically indistinguishable from that of PI [23]. In fact, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine and phosphatidic acid are considered to have similar interactions with aminoglycosides, with $K_d$ values ranging between 3 and 47 μM for the binding of gentamicin [24].

Owing to their oligocationic character at lysosomal pH, aminoglycosides strongly interact with the charged head group of the DMPG molecules, and a direct interaction with the hydrophobic domain has been excluded [24]. However, the binding of these antibiotics to the hydrophilic portion can cause changes in the hydrophobic region and, thus, to the membrane fluidity. These perturbations of the hydrophobic domain can be studied by infrared spectroscopy in the methylene stretching and bending vibration regions where variations in band frequency and shape are observed. Our results reveal that the three antibiotics used in this study induced an increase of the gel to liquid–crystal transition temperature of DMPG. This increase was consistent with a tightening of the lipid network resulting from the neutralization of the negative charges of the lipid head groups. The elimination of the electrostatic repulsion between the lipid molecules favors the Van der Waals interactions between the acyl chains, and these interactions are maximized when the chains are *all trans* and regularly packed. The bilayer is therefore less prone to convert to the fluid, disordered phase when the temperature is raised. Such a stabilization of the ordered gel phase is usually found as a result of the interaction of positively charged peptides [25, 26] or polycations [27] with negatively charged liposomes. Ramsammy and Kaloyanides [28] also found by differential scanning calorimetry that the aminoglycoside gentamicin increases the transition temperature of DPPC:PI liposomes. The changes in transition temperature observed here (Fig. 2) were found to depend on the nature of the aminoglycoside. The largest shift was obtained for kanamycin B, which has the largest charge. As mentioned in the Results, the shifts were small but reproducible.

The carbonyl stretching vibration region gives an insight into the bilayer interface behavior, which is also affected by the addition of the drugs. The decrease of the relative intensity of the hydrogen-bounded carbonyl stretching band can be explained by a reduction of water penetration, as-

suming for now (see below) that these hydrogen bonds involve only water molecules. This, and the stronger hydrogen bonding revealed by a decrease of the frequency of this band, are consistent with a tightening of the lipidic network resulting from charge neutralization of DMPG.

Another interesting feature is the phosphate antisymmetric stretching vibration for which a frequency decrease of 7–8 $cm^{-1}$ was observed when the drugs were added. This effect was larger than that reported by Gurnani *et al.* [21], i.e. a 2-$cm^{-1}$ shift, in their FTIR spectroscopic study of gentamicin effects on phosphatidylinositol bilayers. Mingeot-Leclercq *et al.* [24] found by $^{31}$P-NMR that gentamicin causes a significant restriction in the movement of the phosphate group of the phospholipids. It may seem surprising that the neutralization of the bilayer charges does not yield a larger change of the phosphate band since this group carries the charge of the lipid molecule. If the dissociation constants fall in the range mentioned above, it is clear that essentially all the lipid moelcules are bound under the conditions used in the present study. The massive aggregation observed after adding the drugs to the liposomes confirms an extensive interaction. In fact, it appears that strong electrostatic interactions at the surface of a bilayer do not necessarily yield a modification of the strength of the phosphorous-oxygen bonds involved in the $PO_2^-$ antisymmetric stretching vibration. Hence, the frequency of this band was reported to remain the same after the binding of the myelin basic protein to DMPG [26], or of polymyxin B [25] or polylysine [29] to dipalmitoylphosphatidylglycerol (DPPG). On the other hand, the direct neutralization of the PG head group by a proton is expected to have a dramatic effect on the phosphate vibrations because the two equivalent *bond-and-a-half* bonds of the charged phosphate are replaced by a P=O and a P=OH bond. Lowering the pH from 5.4 to 2 resulted in important changes in the shape and frequency of the phosphate antisymmetric (Fig. 6B, solid line vs dot-dot-long dash) and symmetric (not shown) stretching bands. Clearly, the neutralization by bulky oligo and polycations does not involve a close contact that would result in a change of the *bond-and-a-half* character of the two PO bonds of the $PO_2^-$ moiety. Indeed, polylysine has been shown to simply adsorb on the bilayer surface [29]. McLaughlin and coworkers have shown that the binding of gentamicin and spermine to negatively charged phospholipidic bilayers can be described qualitatively by combining the Gouy-Chapman theory with Langmuir adsorption isotherms [30, 31].

Globally, in the present study, kanamycin B is the drug that exhibited the largest effects since the transition temperature of the liposomes was the highest with kanamycin B and it lowered to the greatest extent the hydrogen-bound carbonyl band frequency. This could be related to the presence of an extra ammonium group on kanamycin B, enabling it to neutralize more efficiently the negative charges on DMPG bilayers because of its higher charge density. It appears also that among the three antibiotics, kanamycin B is the most potent inhibitor of lysosomal phospholipases [32] and has the lowest $LD_{50}$ in mice [33]. Mingeot-Leclercq *et al.* [24] showed by $^{15}$N-NMR spectroscopy that kanamycin B binds more tightly to vesicles and experiences a larger reduction in its mobility than the other two antibiotics [24]. The fact that kanamycin B induces the largest shift of the lipid gel to liquid–crystalline phase transition temperature means that it tightens the lipid head group network more than amikacin or kanamycin A at the same molar ratio, which is consistent with the higher charge density of kanamycin B. This tightening makes the lipid bilayer more resistant to the planar expansion required to go to the fluid phase. However, the frequency of the methylene symmetric stretching (Fig. 2, squares) indicates that amikacin induces the largest decrease of the gauche/trans ratio in the hydrocarbon chains. The fact that this antibiotic gives the smallest increase in transition temperature but the highest ordering of the acyl chains seems contradictory, but it is actually very informative about the lipid–aminoglycoside complex. With amikacin, the lipid head groups get slightly closer together, which results in a small increase in transition temperature, and the hydrocarbon chains pack in a more orderly fashion than for the pure lipid. Amikacin also leads to the largest decrease of the intensity of the hydrogen bonded carbonyl stretching component in the fluid phase (Fig. 4D, dot-long dash). Therefore, amikacin favors a regular packing of the phospholipids and increases the Van der Waals interactions between the straightened, closer hydrocarbon chains. This suggests that amikacin probably lies on the surface of the bilayer, similarly to polylysine [29].

On the other hand, kanamycin B produced the largest increase of the transition temperature but was not as efficient as amikacin in reducing the number of gauche bonds (Fig. 2, squares). This means that although the lipid molecules are, on the average, closer, at least a portion of them is disordered and not *all trans*. Whereas the amikacin-induced changes in the carbonyl stretching band (Fig. 4, dot-long dash) are completely consistent with the antibiotic sticking onto the bilayer surface, the spectrum obtained with kanamycin B (Fig. 4, small dashes) does not agree with that picture. Compared with the pure lipid (Fig. 4, solid line), there was only a small decrease of the intensity of the hydrogen bonded carbonyl stretching component. Interestingly, the frequency of that band was drastically lower than those of the other complexes at any temperature (Fig. 5, filled circles), which could mean that the hydrogen bond partner is different. Furthermore, the frequency of the free carbonyl component (Fig. 4, small dashes) decreased by 4–5 $cm^{-1}$ in the presence of kanamycin B. Frequency shifts of that component indicate either a change in the group environment or a conformational change of the molecule. These perturbations of the carbonyl groups are consistent with a penetration of kanamycin B molecules to a level allowing hydrogen bonding between some of its hydroxyl or amino groups and some lipidic carbonyl groups. Such a localization would also explain why the decrease in the

proportion of gauche bonds is not larger with kanamycin B than with amikacin. It is interesting to compare this view with the results of Mingeot-Leclercq *et al.* [24] who concluded from their conformational analysis that kanamycin B is located between the head groups and close to the interface of a PI bilayer, and tends to adopt an orientation parallel to this interface. They also proposed that amikacin would insert within the bilayer, parallel to its normal. This is hard to reconcile with our results.

Kanamycin A presents intermediate effects on the carbonyl stretching band and on the transition temperature. With fewer charges than kanamycin B and bearing a simple amino group instead of the aminohydroxybutyramido group of amikacin, kanamycin A has a lower charge density than kanamycin B and is less bulky than amikacin, so it is likely to have an intermediate location. Accordingly, this aminoglycoside is not as efficient at increasing the conformational order of the acyl chains (Fig. 2, triangles).

The extent of membrane stabilization by kanamycin B seems to be related to the extent of its inhibitory potential against lysosomal phospholipases. How can a higher stabilization of liposomes be related with a higher nephrotoxicity? As mentioned in the introduction, the accumulation of aminoglycosides in lysosomes is associated with an impairment of the phospholipase activity, resulting in an emergence of myeloid bodies. Carlier *et al.* [32] have shown a direct relationship between the amount of gentamicin, amikacin, or streptomycin bound to PI-containing liposomes and the inhibition of the activity of lysosomal phospholipases towards PC. Lysosomal phospholipases are soluble enzymes bearing positive charges on their surface. An interaction with negatively charged head groups of membrane lipids is required for their activation. Oliver *et al.* [34] showed that the phospholipase activity in dehydrated systems is affected by electrostatic interactions with negative surfaces. They correlated an increased activity of the enzyme with higher PS:PC ratios [34]. Earlier, Mingeot-Leclercq *et al.* [35] and Hostetler and Jellison [36] observed no inhibitory effect on the activity of lysosomal phospholipases when aminoglycosides were added to neutral liposomes of zwitterionic phospholipids. Oliver *et al.* [34] suggested that the wide space separating the head groups in negatively charged liposomes makes the *sn*-2 fatty acid ester bond more accessible to $PLA_2$. Changes in the bilayer organization influence the phospholipase activity. Jain *et al.* [6] performed experiments with lipophilic inhibitors and phospholipase $A_2$. They found that inhibitors that are inserted into the bilayer inhibit indirectly the phospholipase by changing the physical properties of the bilayers, thus making the interaction between the enzyme and the bilayer interface very difficult. It appears to us that the aminoglycosides have the required characteristics to inhibit phospholipase $A_2$: (1) they reduce the electrostatic interaction between the enzyme and the membrane by neutralizing the negative head groups of the latter, and (2) they tighten the membranous network, making the substrate less accessible to the catalytic site. The results obtained in this work support this view. Charge neutralization is a critical determinant in the inhibition of phospholipases. Hydrophilic compounds with only three positive charges, such as streptomycin, are much less potent inhibitors than an aminoglycoside with more positive charges like gentamicin [37]. In addition, the substitution of the N-1-amino function of kanamycin A, or of gentamicin B, by an aminohydroxybutyryl or aminohydroxypropionyl side chain, or the substitution of the position C-1 of gentamicin C2 by a hydroxymethyl group, decreases both the binding to the negatively charged bilayers and the inhibitory potency of the drug towards lysosomal phospholipase activity [32, 37, 38]. Clearly, charge neutralization is not the only parameter involved in the phospholipase inhibition. According to Mingeot-Leclercq *et al.* [24], the energy of interaction between the drug and the phospholipid and the accessibility of the drug to the aqueous phase are also involved. They proposed that the interaction energy of the complex formed by aminoglycosides and phospholipids varies in parallel with the change in inhibitory potency and that the increased drug accessibility to water is associated with a decrease of its inhibitory potency. This is consistent with our proposal that the less toxic amikacin adsorbs on the bilayer surface whereas the more toxic kanamycin B intercalates between the lipid head groups. FTIR spectroscopy proved to be a powerful technique to show how these aminoglycosides affect the conformation of the lipid molecules. It has also been shown to be suitable for the direct study of aminoglycoside effects on the activity of phospholipases [39]. These investigations of phospholipase activity could be revealing concerning the molecular mechanism underlying the nephrotoxicity of aminoglycoside.

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