X-Ray Studies on Phospholipid Bilayers. XIII. Interactions with Gentamicin

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Z. Naturforsch. **48c**, 632–639 (1993); received March 16, 1993

X-ray Diffraction, Phospholipid Bilayer, Gentamicin

This study deals with the structural perturbations that the aminoglycoside antibiotic gentamicin (GENT) can produce to phospholipid bilayers. Two multibilayer systems, one built-up of dimyristoylphosphatidylcholine (DMPC) and the other of dimyristoylphosphatidylethanolamine (DMPE) were allowed to interact with GENT. The experiments were performed in both a hydrophobic and a hydrophilic medium below the phospholipid main transition temperatures. X-ray diffraction techniques were used to determine the extent of the perturbation induced by GENT. The maximum effect was attained when GENT interacted with DMPC in the hydrophobic medium. On the other hand, GENT in aqueous solutions was unable to perturb in any significant extent the structure of the phospholipids under study.

Introduction

Since the isolation of streptomycin by Waksman in 1944, aminoglycoside antibiotics have become one of the major groups of agents used in modern chemotherapy to combat bacterial infections. The main aminoglycosides administered today in clinical practice are gentamicin and tobramycin, two naturally occurring antibiotics, and netilmicin and amikacin, which are semi-synthetic derivatives resistant to many of the bacterial enzymes that inactivate gentamicin and tobramycin [1]. The aminoglycoside antibiotics are generally hydrophilic basic compounds, active against a broad spectrum of both Gram-positive and Gram-negative bacteria [2].

Gentamicin (GENT), in common with the other aminoglycoside antibiotics, exhibits some interesting biophysical properties. It does not transport to any significant degree across the membrane of the gastrointestinal tract given its character of a highly polar cation [3]. In contrast, GENT does cross some specialized membranes; *e.g.*, it penetrates the aqueous humor of the eye, passes the placental barrier into the amiotic fluid, and transports across the otic membrane. Furthermore, since the site of its antibacterial action is at the ribosome level – where it inhibits the synthesis of proteins – it clearly must penetrate the bacterial membrane [4]. However, the mechanism by which GENT transports across bacterial and other membranes has not been firmly established. Its solubility characteristics and its negligible oil-water partition coefficient preclude dissolution or partitioning into lipoidal membranes. Its relatively large molecular size precludes transport through water-filled pores or channels. Therefore, it appears that its transport must involve, at least as a first step, some specific interactions with a cell membrane component [4].

The untoward effects of GENT are similar to those of other aminoglycoside antibiotics. The most important and serious side effects of its use are nephrotoxicity and irreversible ototoxicity [3]. While the mechanism of their toxicity seem to be rather complex, a number of attempts have been made to create simple model systems to study or evaluate the toxicity of aminoglycoside antibiotics. These model systems are based on the *in vitro* interaction of the drugs with receptor-like binding sites such as mucopolysaccharides [5], phospholipids [6] and even nonphysiological compounds [7]. Only phospholipids have been strongly implicated as potential targets for the aminoglycoside antibiotics in the kidney and the inner ear *in vivo* [8], and considerable evidence has been presented that phosphatidylinositol serves as a specific binding site [9, 10]. Indeed, effects of aminoglycoside antibiotics on membrane lipids have been reported [11]. Specifically, aminoglycosides competitively bind to Ca^{2+} binding sites on a model phospholipid membrane [12], increase the surface pressure of lipid monomolecular films [4], affect the electrophoretic mobility of the phospholipid liposomes [13], and the addition of GENT to one side of a bi-

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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/0700–0632 $ 01.30/0

layer lipid membrane results in a potential difference across the membrane [11].

For these reasons it was thought of interest to study the interaction of GENT (whose schematic structure is shown in Fig. 1) with a model system consisting of multibilayers built-up of dimirystoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidylcholine (DMPC). These phospholipids are representative of cephalins and lecithins, which are mostly and respectively located in the inner und outer monolayers of the erythrocyte membranes [14]. Their structures under different hydrations below their main transition temperatures have been reported [15–16]. Chemically, they only differ in their terminal amino groups, being $^{+}NH_3$ in DMPE and $^{+}N(CH_3)_3$ in DMPC. Their bilayer structures are very similar in the dry crystalline phases (Lc). In fact, both have the hydrocarbon chains mostly parallel and completely extended with their polar groups lying perpendicularly to them. However, DMPE molecules pack more tightly than those of DMPC. This effect, due to the smaller polar group and higher effective charge of DMPE, results in a very stable bilayer structure which is not significatively perturbed by the addition of water [16]. However, the gradual hydration of DMPC under the same conditions results in water molecules occupying the highly polar interbilayer spaces. As a consequence, there is an increase in its bilayer separation [15] and the phospholipid undergoes the phase transition $Lc \leftrightharpoons L\beta' \leftrightharpoons P\beta' \leftrightharpoons L\alpha$. Lc denotes the crystalline phase, $L\beta'$ the gel phase, $P\beta'$ the rippled gel phase and $L\alpha$ the liquid crystalline phase, the latter being generally present at high temperatures.

These bilayer systems and the X-ray diffraction methods have already been used in this laboratory to study their interactions with the antibiotics chlortetracycline [17] and chloramphenicol [18], as well as with other chemicals of biological interest [19–21]. The results have allowed an insight about the way these compounds might perturb the structure of cell membranes.

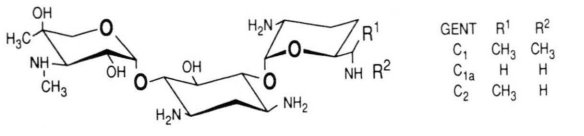

Fig. 1. Schematic structure of GENT. It consists of two aminosugars connected through glycosidic bonds to a hexose in a central position. GENT is a mixture of three components (C_1, $C_{1\alpha}$ and C_2), whose biological activities are essentially identical.

Materials and Methods

Synthetic DMPE from SIGMA (Lot 88F-8365, A Grade, MW 635.9), DMPC from SIGMA (Lot 46F-8410, A Grade, MW 677.9) and GENT from SIGMA (Lot 50H-7712, under the form of gentamicin sulfate, purity 614 μg gentamicin per mg solid. Molar concentrations were calculated using a molecular mass of 754) were used without further purification. Ethylene glycol from Fluka (P.A. grade), chloroform from Merck (Uvasol, spectroscopic grade) and methanol from Merck (Uvasol, spectroscopic grade) were the solvents. Powder mixtures of DMPE:GENT and DMPC:GENT were prepared in the molar ratios of 10:1, 5:1 and 1:1. Each mixture was dissolved in chloroform: methanol:ethylene glycol 3:1:1 and left for two days at room temperature in order to facilitate their possible molecular interactions. The solvent was then removed by blowing warm air onto each solution. The same procedure was followed with pure samples of DMPE, DMPC and GENT. The resulting residues were in the form of dry and crystalline powders except those of the DMPC:GENT mixtures, which had the appearance of wet pastes. These were later dried by heating at 104 °C over P_2O_5 under vacuum for 4 h. The samples thus prepared were placed into low absorbing 0.7 mm diameter X-ray glass capillaries and sealed. They were X-ray diffracted in Debye-Scherrer cameras of 114.6 mm diameter and flat-plate cameras with 0.25 mm inner diameter glass collimators [16], provided with rotating devices.

Hydrated samples were prepared in 1.5 mm diameter X-ray glass capillaries, each containing about 3 mg of DMPE or DMPC. To each capillary it was added about 140 μl of a) pure water; b) 1.0 mM GENT; c) 10.0 mM GENT and d) 100.0 mM GENT, and then sealed. They were X-ray diffracted 48 h after preparation in flat-plate cameras. Specimen-to-film distances were either 8 or 14 cm, standardized by sprinkling calcite powder on the capillaries surface. Ni-filtered CuKα radiation from a Philips PW 1140 X-ray generator was used. The relative intensities of the reflections were measured from films in a Joyce-

Loebl MK III CS microdensitometer connected to an Acer 915 microcomputer. All the experiments with aqueous solutions were carried out at about 17 ± 2 °C, which is below the main transition temperature of each phospholipid unter study.

Results

The molecular interactions of GENT with multibilayers of the phospholipids DMPC and DMPE were studied by X-ray diffraction techniques. The patterns were obtained from a) dry samples of each phospholipid with GENT in the molar ratios of 10:1, 5:1 and 1:1 recrystallized from hydrophobic solutions (chloroform:methanol:ethylene glycol 3:1:1), and b) mixtures of each phospholipid in their crystalline phases with 1.0 mM, 10.0 mM, 100.0 mM GENT aqueous solutions. These patterns were compared with those of the pure GENT and the corresponding phospholipid obtained under the same physicochemical conditions. The results are shown in Tables I to IV and Fig. 2 to 5. Table I presents the interplanar spacings and the relative intensities of the reflections produced by dry samples of DMPC and its mixtures with GENT, while their diffractograms are compared in Fig. 2. The analysis of these results indicated that the X-ray pattern of DMPC was perturbed by increasing concentrations of GENT. In fact, at such a small DMPC:GENT molar ratio as of 10:1, most of the wide-angle reflections of DMPC were absent, including those strong ones of 4.29 Å, 4.10 Å and 3.88 Å. Instead, a strong new reflection of 4.2 Å showed up, together with two very weak reflections of 6.94 Å and 4.79 Å. At a molar ratio of 1:1, only four reflections remained, being their intensities weaker than when they were observed at lower concentrations of GENT. On the other hand, the first-order reflection of 55.2 Å observed in the low-angle region of DMPC, as well as its 2nd, 3rd and 4th order accompanying reflec-

Table I. Comparison of observed interplanar spacings (do) and relatives intensities (Io rel) of DMPC and of its mixtures with GENT in the molar ratios of 10:1, 5:1 and 1:1 obtained from dry samples recrystallized from chloroform:methanol:ethylene glycol 3:1:1 (a, b, c).

DMPC		DMPC:GENT 10:1		DMPC:GENT 5:1		DMPC:GENT 1:1	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
55.2*	5115	55.5*	4866	55.0*	4612	55.5*	3045
27.4	55	27.7	42	27.5	49	–	–
18.6	25	18.7	24	18.7	27	–	–
13.6	156	13.8	184	13.8	163	13.6	52
9.33	31	9.29	23	9.31	8	–	–
8.38	8	–	–	–	–	–	–
7.25	1	–	–	–	–	–	–
–	–	6.94	2	–	–	–	–
6.25	73	–	–	–	–	–	–
5.83	25	–	–	–	–	–	–
5.27	19	–	–	–	–	–	–
4.99	9	–	–	–	–	–	–
–	–	4.79	1	4.78	1	4.79	1
4.66	61	–	–	–	–	-	–
4.29	309	–	–	–	–	–	–
–	–	4.24	963	4.21	794	4.18	288
4.10	775	–	–	–	–	–	–
3.88	101	–	–	–	–	–	–
3.72	3	–	–	–	–	–	–
3.35	1	–	–	–	–	–	–
3.19	32	–	–	–	–	–	–
3.02	2	–	–	–	–	–	–

(a) Recrystallized GENT gave an amorphous pattern as can be observed in Fig. 2.
(b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras D = 8 and 14* cm.
(c) Additional reflections with spacings below 3.0 Å were also observed.

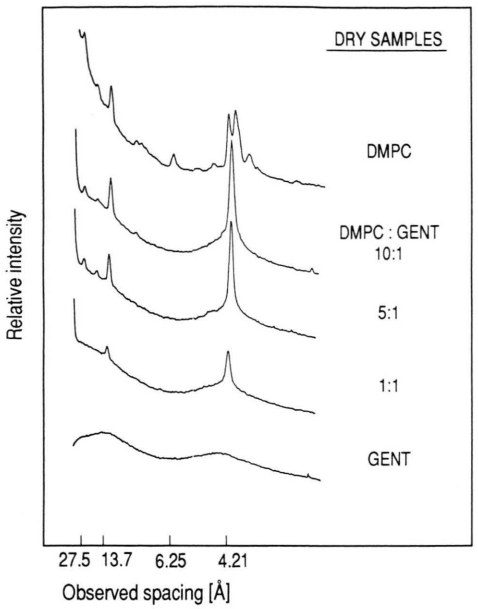

Fig. 2. Microdensitograms from X-ray diffraction diagrams of dry specimens recrystallized from chloroform: methanol:ethylene glycol 3:1:1. Flat-plate cameras (D = 8 cm).

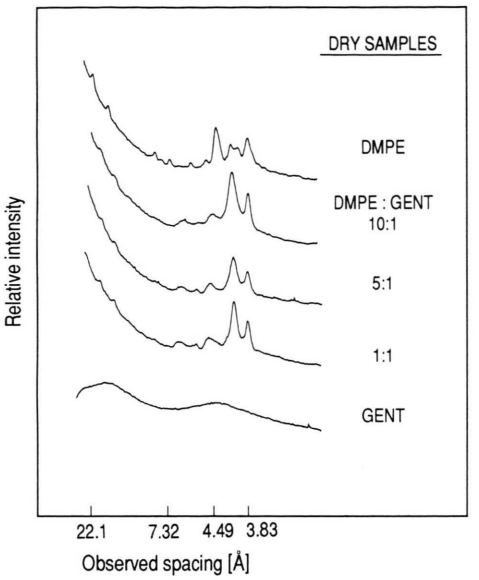

Fig. 3: Microdensitograms from X-ray difraction diagrams of dry specimens recrystallized from chloroform: methanol:ethylene glycol 3:1:1. Flat-plate cameras (D = 8 cm).

tions, were present in all its patterns without significant changes except in their relative intensities.

Table II and Fig. 3 show the results obtained from dry samples of DMPE, and of its molar mixtures with GENT. It has been reported that this phospholipid presents two polymorphic forms when it is recrystallized from chloroform:methanol solutions [17]. One phase (Lc_1) is obtained when these solvents are in a 3:1 v/v ratio; its bilayer repeat of about 52 Å indicates that the hydrocarbon chains are extended and parallel to the bilayer normal [16]. The other phase of DMPE (Lc_2) can be obtained by its recrystallization from chloroform:methanol 1:3. In this case, the bilayer repeat is of about 44 Å because the hydrocarbon chains are now tilted by nearly 30°. The results obtained in the present investigation showed that DMPE, when recrystallized from chloroform: methanol:ethylene glycol 3:1:1, showed the Lc_2 tilted form, proved by the 44.2 Å bilayer repeat of DMPE. However, the X-ray patterns of all DMPE mixtures with GENT presented DMPE in its Lc_1 extended form. On the other hand, the increasing proportion of GENT in the mixtures did not significatively affect DMPE bilayer width of about 52 Å.

Fig. 4 and Table III respectively show the X-ray patterns and the interplanar spacings obtained two days after DMPC in its crystalline phase was

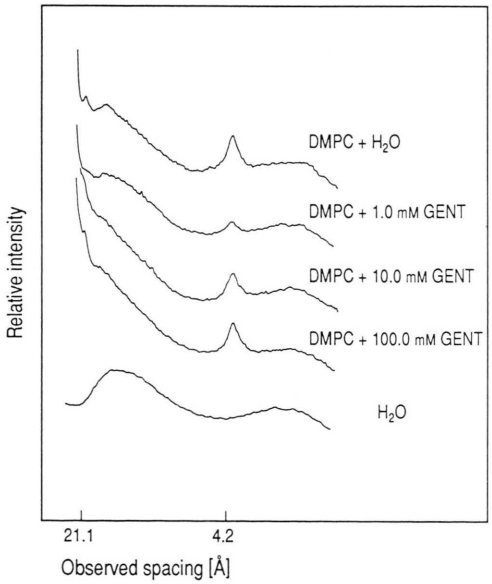

Fig. 4. Microdensitograms from X-ray diffraction diagrams of aqueous mixtures of DMPC. Flat-plate cameras (D = 8 cm).

Table II. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE and of its mixtures with GENT in the molar ratios of 10:1, 5:1 and 1:1 obtained from dry samples recrystallized from chloroform:methanol:ethylene glycol 3:1:1 (a, b, c).

DMPE		DMPE:GENT					
		10:1		5:1		1:1	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
—	–	51.7*	3617	52.0*	1747	51.5*	3621
44.2*	1435	–	–	–	–	–	–
–	–	25.4	3	25.3	7	25.5	12
22.1	41	–	–	–	–	–	–
–	–	17.4	28	17.4	26	17.4	25
14.9	26	–	–	–	–	–	–
12.7	3	12.9	25	13.0	28	12.9	26
11.3	1	–	–	–	–	–	–
–	–	10.1	6	10.0	4	10.0	7
9.09	1	–	–	–	–	–	–
7.32	25	–	–	–	–	–	–
–	–	7.20	2	7.20	4	7.18	10
6.80	13	–	–	–	–	–	–
6.33	21	–	–	–	–	6.30	1
–	–	5.92	70	5.95	61	6.03	1
5.86	1	5.78	70	5.80	61	5.86	124
5.31	21	–	–	–	–	–	–
–	–	5.10	6	5.13	26	5.11	21
4.80	24	–	–	–	–	–	–
–	–	4.68	102	4.69	87	4.70	185
4.49	408	–	–	–	–	–	–
–	–	4.22	3	4.22	5	4.26	10
4.17	163	4.13	956	4.10	530	4.08	630
4.01	129	–	–	–	–	–	–
3.82	332	3.83	381	3.83	228	3.83	256
3.65	7	3.65	1	3.65	1	3.64	1
3.40	6	3.40	1	3.40	1	3.40	13
3.14	7	3.17	1	3.16	1	3.16	1

(a) Recrystallized GENT gave an amorphous pattern as can be observed in Fig. 3.
(b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14* cm.
(c) Additional reflections with spacings below 3.0 Å were also observed.

Table III. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPC specimens with water and GENT aqueous solutions (a, b).

DMPC + H_2O		DMPC + 1.0 mM GENT		DMPC + 10.0 mM GENT		DMPC + 100.0 mM GENT	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
64.4*	3173	64.6*	791	64.3*	2191	63.0*	3404
32.2	1004	32.3	769	32.2	1041	31.4	1157
21.2	28	21.4	16	21.1	27	20.8	23
13.1	22	13.0	1	12.9	26	12.5	21
4.20	498	4.20	199	4.20	392	4.20	500

(a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14* cm.
(b) The samples were left for 2 days before being X-ray diffracted.

mixed and allowed to interact with water and GENT aqueous solutions. The observed results indicated, first, that as expected the pattern of DMPC changed after the addition of water in excess from that showed by its dry crystalline phase in Fig. 2. In fact, there is an increase of its bilayer repeat from 55.2 Å to 64.4 Å. Besides, it only showed four reflections, all orders of the 64.4 Å period, and one strong reflection of 4.2 Å. On the other hand GENT, even in its most concentrated solution, did not significatively affect the pattern of DMPC. The only noticeable effect was a gradual shortening of its bilayer width from 64.4 Å when mixed with pure water, down to 63.0 Å in the presence of 100.0 mM GENT solution.

Finally, Fig. 5 and Table IV present the results obtained when GENT aqueous solutions were allowed to interact with DMPE in the same conditions as described for DMPC. First, it can be observed that the X-ray pattern of DMPE in the presence of water did not change too much with respect to that obtained from its dry crystalline form. In fact, it remained essentially the same inclined Lc_2 phase, except in that its bilayer repeat slightly increased from 44.2 Å when dry to 45.6 Å, and some of its weaker reflections were absent in the humid form. On the other hand, GENT did not affect in any significant extent the X-ray pattern of DMPE, even in its most concentrated solution.

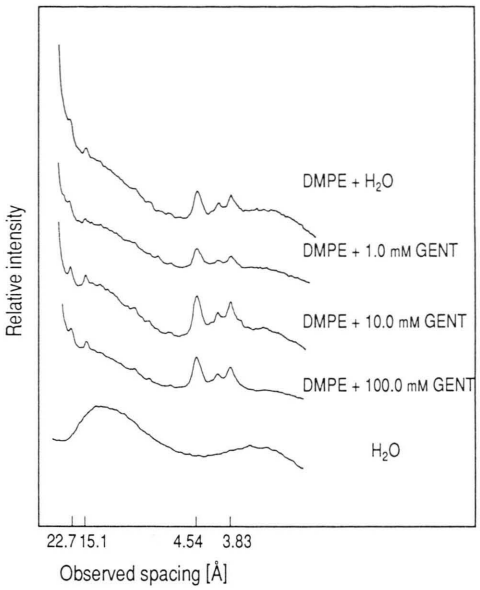

Fig. 5. Microdensitograms from X-ray diffraction diagrams of aqueous mixtures of DMPE. Flat-plate cameras (D = 8 cm).

Discussion

Model systems consisting of synthetic phospholipids have been a valuable tool for obtaining information about the structure and physicochemical characteristics of the lipid bilayers of cell membranes. They are also been used to study how ther-

Table IV. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE specimens with water and GENT aqueous solutions (a, b).

DMPE + H_2O		DMPE + 1.0 mM GENT		DMPE + 10.0 mM GENT		DMPE + 100.0 mM GENT	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
45.6	3294	45.5	2409	45.6	3871	45.6	1746
22.5	36	22.7	41	22.8	40	22.8	34
15.1	48	15.1	38	15.1	43	15.1	38
11.4	1	11.4	1	11.4	1	11.4	1
7.30	19	7.29	26	7.29	20	7.29	16
6.39	26	6.35	10	6.35	15	6.35	13
5.35	20	5.38	10	5.38	21	5.38	12
4.54	355	4.54	212	4.54	427	4.54	345
4.08	127	4.07	54	4.07	111	4.07	98
3.83	258	3.83	170	3.82	332	3.83	232

(a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 8* cm.
(b) The samples were left for 2 days before being X-ray diffracted.

apeutical drugs and other chemicals of biological interest might affect their integrity and functionality, information that can help to understand the toxic mechanisms of these compounds. In the present study the aminoglycoside antibiotic GENT was made to interact with DMPE and DMPC multibilayers. Each one represents a type of phospholipid which is respectively found in the inner and outer monolayers of many biological membranes, particularly that of the human red blood cell. Their interactions with GENT were studied by X-ray diffraction below their main transition temperatures under two different physicochemical conditions. In the first GENT, which presents a hydrophilic and cationic character, was made to interact with each phospholipid in a hydrophobic medium. This could be attained by dissolving both the phospholipid under study and GENT in various molar ratios, in chloroform:methanol:ethylene glycol 3:1:1. After 48 h the solvent was removed by evaporation and each phospholipid:GENT molar mixture, under the form of a dry and crystalline powder, was X-ray diffracted. Their X-ray patterns were then compared with those of the single phases of each phospholipid. GENT, which is a mixture of three type of molecules (Fig. 1), could not crystallize giving, therefore, amorphous patterns (Fig. 2 and 3). The results obtained indicate that GENT was able to perturb to different degrees the bilayer arrangements of both phospholipids. In the case of DMPE, the observed effects were rather mild. In fact, it was only observed that DMPE, in the presence of GENT, recrystallized in the extended Lc_1 form instead of the Lc_2 tilted one, which is the phase under which DMPE recrystallized from chloroform:methanol:ethylene glycol 3:1:1. This phase transition of DMPE is most likely due to a change in the physicochemical properties of the solvent after the dissolution of the highly polar gentamicin sulphate rather than to a molecular interaction between GENT and DMPE. This is confirmed by the fact that higher concentrations of GENT did not significatively affect the described result. On the other hand, it should be mentioned that nearly similar effects on DMPE have been described for the amphipathic antibiotics chlortetracycline [17] and chloramphenicol [18].

The changes produced by GENT to DMPC bilayers in the same hydrophobic medium are indeed more pronounced than those observed in DMPE. As it can be seen in Fig. 2 and Table I, only one molecule of the antibiotic in ten molecules of DMPC was enough to perturb in a significant extent the phospholipid bilayer structure. This structural alteration, which increased with higher proportions of GENT in the mixtures, produced a phase transition from the crystalline Lc form of DMPC to a more fluid phase. The fact that the bilayer repeat remain unchanged at about 55.5 Å despite the gradual incorporation of GENT, points to a deep penetration of the antibiotic into the hydrophobic core of DMPC resulting in its fluidization. The appearance of the 4.2 Å reflection in all DMPC:GENT mixtures proves that the hydrocarbon chains of the lipid become hexagonally arranged. In fact, this reflection, present in lecithin:water mixtures below their main transition temperatures (β and β′ phases) arises from the stiff and fully extended hydrocarbon chains organized with rotational disorder in a hexagonal lattice [22–23]. The results observed in the present study are remarkable as these structural perturbations were produced by GENT in the absence of water and below the main transition temperature of DMPC. A somewhat similar result was also observed in the case of chlortetracycline [17].

In the second type of physicochemical condition, GENT was made to interact with both phospholipid bilayers in a hydrophilic medium. For this purpose, aqueous solutions of GENT in several concentrations were separately mixed with DMPC and DMPE in their crystalline Lc phases. The observed results indicate that GENT does not interact with DMPC despite the open and accessible packing arrangement of the phospholipid under the influence of water, quite suitable for the interaction of the cationic aminoglycoside antibiotic with the phosphate charged groups of DMPC. In view of this result, it was not surprising that GENT neither interacts with DMPE bilayers given their closer and tighter packing.

The results obtained in a hydrophilic medium of the present study agree with those reported in the literature. In fact, it has been published that GENT, as well as other aminoglycoside antibiotics, does not interact with lecithin neither phosphatidylethanolamine bilayers [4, 9, 10]. Nevertheless, it has been found that the addition of GENT to one side of a lecithin bilayer membrane results

in a potential difference, which has been explained as due to the adsorption of GENT bearing four positive charges onto the bilayer surface. This result, however, is strongly dependent on the pH and ionic strength of the medium [11]. On the other hand, experimental evidences indicate that there are strong affinities between GENT and negatively charged phospholipids such as phosphatidylinositol [9, 24], phosphatidylserine and phosphatidic acid [25]. These interactions have been used to explain the nephrotoxicity induced by aminoglycoside antibiotics to humans and animals. In fact, it has been found that the activity of lysosomal phospholipases towards phosphatidylcholines included in unilamellar liposomes is markedly increased upon the addition of phosphatidylinositol in the bilayers [26]. The aminoglycoside antibiotics then, by binding to and neutralizing the negative charges of the membrane, would inhibit phospholipase activity [25], a key event in the sequence of reactions leading to aminoglycoside nephrotoxicity [10].

Acknowledgements

Research grants from FONDECYT (1930504) and the University of Concepción (92.13.94-1) are gratefully acknowledged.

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