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ULTRASTRUCTURAL, PHYSICO-CHEMICAL AND CONFORMATIONAL STUDY OF THE INTERACTIONS OF GENTAMICIN AND BIS(BETA-DIETHYLAMINOETHYLETHER)HEXESTROL WITH NEGATIVELY-CHARGED PHOSPHOLIPID LAYERS

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Abstract—Aminoglycoside antibiotics such as gentamicin, which are fully hydrophilic, and cationic amphiphilic drugs such as bis(beta-diethylaminoethylether)hexestrol (DEH), are both known to inhibit lysosomal phospholipases and induce phospholipidosis. This enzymatic inhibition is probably related to the neutralization of the surface negative charges on which the lysosomal phospholipases A1 and A2 are dependent to express fully their activities (Mingeot-Leclercq *et al.*, *Biochem Pharmacol* **37**: 591–599, 1988).

Using negatively charged liposomes, we show by ^{31}P NMR spectroscopy that both gentamicin and DEH cause a significant restriction in the phosphate head mobility and, in sonicated vesicles, the appearance of large bilayer structures. Both DEH and gentamicin increased the apparent size of sonicated negatively charged liposomes (but not of neutral liposomes) as measured by quasi-elastic light scattering spectroscopy. Examination of replicas from freeze-etched samples, however, revealed that gentamicin caused aggregation of liposomes, whereas DEH induced their fusion and the formation of intramembranous roundly shaped structures. Only DEH caused a significant decrease of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, a fluorescent lipid-soluble probe. In addition, DEH, but not gentamicin, interfered with the bilayer to hexagonal phase transition occurring in dioleoyl- and dielaidoylphosphatidylethanolamine liposomes upon warming, and caused the appearance of an isotropic signal suggestive of the formation of inverted micelles. In computer-aided conformational analysis of the molecules at a simulated air–water interface, gentamicin was shown to display a largely-open crescent shape. When surrounded by phosphatidylinositol molecules, it remained as such at the interface which it locally mis-shaped, establishing close contact with the negatively charged phospho groups. In contrast, DEH could be oriented perpendicularly to the interface, with its two cationic groups associated with the phospho groups, and its phenyl- and diethylethanediyl moieties deeply inserted between and interacting with the aliphatic chains. Thus, although both agents cause lysosomal phospholipases inhibition, the differences in their interactions with negatively-charged bilayers is likely to result in a different organization of the phospholipids accumulated *in vivo*, which could lead to different toxicities.

Lysosomal phospholipidosis is a conspicuous side effect of two types of drugs with largely different physico-chemical properties, namely the aminoglycoside antibiotics such as gentamicin, which are totally hydrophilic polycationic compounds [1], and the so-called cationic amphiphilic drugs, which belong to various pharmacological groups but have in common to carry one or two aminogroups together with a bulky hydrophobic moiety [2]. A typical example of the second type of drug is the formerly used antianginal drug bis(beta-diethylaminoethylether)hexestrol (DEH). Cationic amphiphilic drugs readily diffuse through membranes and therefore enter many organs. They accumulate in lysosomes [3], probably by ion-trapping and by autophagic sequestration of the intracellular membranes which they bind to [4, 5]. Aminoglycosides are non-diffusible drugs, but they can enter cells by endocytosis and also reach high intralysosomal concentrations [6–11].

Both cationic amphiphiles and aminoglycosides inhibit the activities of lysosomal phospholipases towards phosphatidylcholine included in negatively-charged lipid layers *in vitro* [12–15]. We recently proposed [16] that the drugs actually reduce the availability of the surface negative charges which lysosomal phospholipases require to act fully on phosphatidylcholine present in a bilayer, even

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though cationic amphiphiles [17], but not aminoglycosides [15, 16], are also able to inhibit lysosomal phospholipases towards phospholipids included in neutral bilayers. Binding of aminoglycosides and cationic amphiphiles to negatively charged phospholipid layers has been demonstrated [13, 18], and an analysis of the effects of both types of drugs towards negatively charged bilayers seemed therefore particularly important, especially since most natural membranes are negative [19]. We have therefore undertaken and report here on a comparative biophysical description of the interactions between negatively charged phospholipid bilayers and gentamicin or DEH, taken as typical examples of an aminoglycoside antibiotic and of a cationic amphiphilic drug, respectively. Investigations were carried out at pH 5.4 to mimick in this respect the conditions prevailing in lysosomes [20, 21].

MATERIALS AND METHODS

Preparation of liposomes

Unless stated otherwise, liposomes were prepared as described earlier [13, 16] with cholesterol, egg-yolk phosphatidylcholine, bovine brain sphingomyelin and wheat germ phosphatidylinositol in a constant molar proportion of 5.5:4:5:2. Except for lipid polymorphism studies and measurement of membrane fluidity, these liposomes were prepared in 4 mM acetate buffer pH 5.4. The total lipid concentration of the preparations was varied according to the experiment performed and is indicated below.

Analysis of liposomes dimensions and morphology

These studies were performed on sonicated liposome preparations containing 10 mg of total lipids per ml. The apparent average diameter of the liposomes was first evaluated by quasielastic light scattering spectroscopy [22] using a Coulter® Nano Sizer™ (Coulter Electronics Ltd., Luton, Beds.), at an angle of 90°, and using monodisperse latex particles of 100 and 800 nm diameter (Dow Chemical Co., Midland, MI) for control. The real, individual diameter of particles was then measured by observation of replicas of freeze-etched samples in the electron microscope [23]. Liposomes were first spray-frozen in the absence of cryoprotectants [24]. We checked by light scattering spectroscopy that spray-freezing did not change the apparent average size of the liposomes in our samples. Freeze-fracturing was made at −100° and at a pressure of 4.10^{-6} Torr with a Balzers model BA 360 M apparatus (Balzers AG, Balzers, Lichtenstein) and examined in a Philips EM microscope at 60 kV. Morphometric analysis of the liposome profiles was made on seven pictures taken at random from each specimen at a microscope magnification setting of 34,000×. The accurate magnification was determined for each series of samples by comparison with a grating replica (2160 lines per mm). For each sample, 300–900 liposome profiles were measured with a Zeiss TGZ 3 particle size analyzer [25] and their diameter recorded in 4.3 nm class intervals.

Measurement of membrane fluidity

The method of Shinitzky and Barenholz [26] was followed to estimate membrane fluidity. The liposomes, prepared in 40 mM acetate buffer pH 5.4, were of the same composition as that used for the analysis of particle dimension, but their concentration was reduced to 0.25 mg lipids/ml, and the sonication step was omitted to obtain large multilamellar vesicles. In addition, the organic solvents in which the lipids were dissolved prior to the preparation of the liposomes contained a fluorescent lipid-soluble probe, 1,6-diphenyl-1,3,5-hexatriene (1 mol per 500 mol lipids). After addition of the drug to the liposomes, the temperature was gradually increased from 288°K to 328°K over approx. 1 hr, while the samples were illuminated with a polarized light beam at 357 nm. Light emission was measured at 428 nm in the planes parallel and perpendicular to the plane of the polarized excitation light, using an Aminco spectrofluorometer SLM-4800 equipped with a Glan-Thomson polarizer. Results were expressed as the degree of fluorescence polarization.

^{31}P-nuclear magnetic resonance spectroscopy

(a) *Chemical shift anisotropy.* These studies were performed with unsonicated and sonicated liposomes of exactly the same composition and concentration as those used for the analysis of particles dimension and morphology. The liposome preparations were placed in 10 mm diameter NMR tubes. A WM 250 Bruker Fourier Transform NMR spectrometer, operating at 101.3 MHz for ^{31}P observation, was used with temperature control, deuterium "lock" (20% D_2O added to the sample) and proton decoupling (5 W). Accumulated free induction decays were obtained from a number of transients up to 20,000 using 90° r.f. pulse with a 0.5 sec interpulse time.

(b) *Lipid polymorphism studies.* These studies were performed on unsonicated liposomes made of dioleoylphosphatidylethanolamine and dielaidoylphosphatidylethanolamine (1:1) according to Cullis and de Kruijff [27] at a final concentration of 5.5 mg lipids/ml in 40 mM acetate buffer pH 5.4. The drugs were added at a final drug–lipid ratio of 1:3 (w:w). The samples were brought at increasing temperatures from 286°K to 314°K by steps of 4°K, and spectra were accumulated at each step over a period of 60 min. The other experimental conditions were the same as described above.

Computer-aided semi-empirical conformational analysis

The general procedure used to determine the conformation of the isolated molecules of drugs and of phosphatidylinositol at a simulated lipid–water interface was the same as that described earlier [28–30]. In addition, the conformation of DEH was also calculated in a totally hydrophobic environment, by setting the dielectric constant to 2.

Assembly of drugs and phosphatidylinositol to form mixed drug–lipid monolayers was then made as described in Ref. 31. Compared to our earlier approach [28, 32], this procedure uses a hypermatrix to store the energies and the coordinates of each molecule of phosphatidylinositol assembled in succession around gentamicin. This procedure is faster, allows a more accurate analysis of the molecular

interactions and, therefore, a more precise determination of the positions of lowest energy. This procedure has recently been successfully applied to the analysis of the interactions of peptides and proteins with membrane bilayers [33]. All calculations were performed on an Olivetti M28 microcomputer equipped with an Intel® 80287 arithmetic co-processor, using the PC-MSA® (Molecular Structure Analyses) and the PC-TAMMO® (Theoretical Analysis of Molecular Membrane Organization) procedures. Graphs were drawn with the PC-MGM® (Molecular Graphics Manipulation) program. Further information on these procedures and programs, and on their availability can be obtained from their author (R.B.).

Materials

Gentamicin (sulphate salt) was supplied by Schering Corporation, U.S.A. (Kenilworth, NJ) as "Gentamicin reagent solution" for *in vitro* and investigational use. As for all commercial preparations of gentamicin, it consisted of a mixture of 3 main components, gentamicin C1, C1a and C2, in a molar ratio of approx. 30:30:40. These components only differ by the methylation of the C'6 and/or the N'6 atoms (see Fig. 5). Previous studies have shown that the interactions of these three components with phosphatidylinositol and their inhibitory activity towards lysosomal phospholipases *in vitro* are similar [15, 32]. DEH was a kind gift from the Upjohn Company (Kalamazoo, MI). The natural glycerophospholipids (egg yolk phosphatidylcholine and wheat germ phosphatidylinositol (grade 1)) were purchased from Lipid Product (Nr. Redhill, U.K.), 1,6-diphenyl-1,3,5-hexatriene, bovine brain sphingomyelin and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Dioleoylphosphatidylethanolamine and dielaidoylphosphatidylethanolamine came from Avanti Polar Lipids (Birmingham, AL). Other reagents (analytical grade) were supplied by E. Merck, AG (Darmstadt, F.R.G.).

RESULTS

Chemical shift anisotropy variations in function of temperature

Since gentamicin and DEH are both cationic at pH 5.4, they may interact with the negatively-charged phospho group in phospholipids, which can be measured by examining the decrease in effective chemical shift anisotropy ($\Delta\sigma$) [34] observed by ^{31}P NMR spectroscopy of liposomes upon warming. For this particular study, we used unsonicated liposomes since the ^{31}P-NMR spectra of sonicated liposomes does not allow to study this parameter [35]. Figure 1 shows that the effective chemical shift anisotropy steadily decreased when control liposomes were warmed from 298°K to 348°K. This decrease was significantly lower in the presence of either gentamicin or DEH (added at an equinormal concentration). Except at 328°K, the differences observed between gentamicin- and DEH-treated liposomes were not significant. Addition of 2-D-glucosamine (4.2 mM) had no effect as compared to control liposomes.

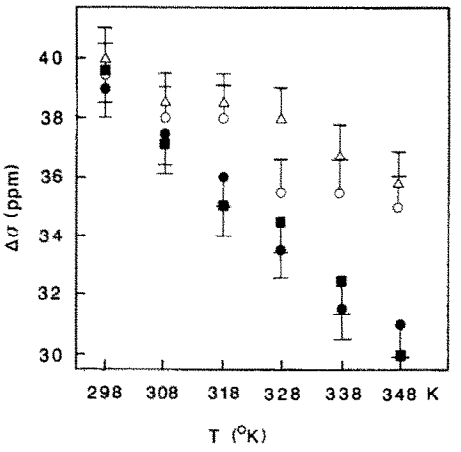


Fig. 1. Variation of the effective chemical shift anisotropy ($\Delta\sigma$, the chemical shift between the high field peak and the low field shoulder of the ^{31}P-NMR broad spectra) of unsonicated, negatively charged liposomes (cholesterol : phosphatidylcholine : sphingomyelin : phosphatidylinositol; molar proportion 5.5 : 4 : 5 : 2) as a function of the temperature. The liposomes (10 mg lipids/ml) were prepared in 4 mM acetate buffer pH 5.4. ●, control liposomes; ■, ○, △, liposomes incubated 1 hr at 37° with 4.2 mM glucosamine, 0.32 mM gentamicin or 0.8 mM DEH, respectively. Each series of samples was successively examined at increasing temperature and recordings were made over approx. 1 hr at each of the temperatures indicated in the abscissa. The vertical bars give the SD of the recordings made with four independent liposomes preparations.

Fluorescence polarization measurements

The influence of gentamicin and DEH on the fluidity of the aliphatic chains of the phospholipids was then examined by measuring the degree of polarization of the light emitted by 1,6-diphenyl-1,3,5-hexatriene inserted into the bilayer, upon excitation by a polarized light beam, and its decrease over warming. As shown in Table 1, the polarization of the emitted light was lower in the presence of DEH

Table 1. Fluorescence polarization of the light emitted by 1,6-diphenyl-1,3,5-hexatriene embedded in unsonicated liposomes upon excitation by a beam of polarized light

	Polarization observed at	
	298°K	310°K
Control	0.3672 ± 0.0048	0.3169 ± 0.0069
+ Glucosamine	0.3631 ± 0.0054	0.3126 ± 0.0055
+ Gentamicin	0.3646 ± 0.0063	0.3103 ± 0.0062
+ DEH	0.3561 ± 0.0051*	0.3006 ± 0.0010*

* Significantly different from control ($P \leq 0.05$). The other values are not significantly different from controls.

Liposomes were prepared as in Fig. 1, except that their concentration was set at 0.25 mg lipids/ml, and incubated 1 hr with glucosamine, gentamicin or DEH, at a drug/lipids ratio similar to that used in Fig. 1. Results are expressed as the degree of fluorescence polarization.

$P = ((I_{paralll.}/I_{perpend.}) - 1)/((I_{paralll.}/I_{perpend.}) + 1)$, as defined by Shinitzky and Barenholz [43]. Results are given as means (±SD) of 3 independent experiments.

Table 2. Influence of gentamicin or DEH on the apparent size of negatively charged and neutral liposomes, as measured by quasi-elastic light scattering spectroscopy (Coulter®, Nanosizer™)

Type of liposomes	Drug*	Diameter (nm)†	No. of different liposomes preparations examined
Negative‡	None	98 ± 9	8
	0.065 mM gentam.	94 ± 10	1
	0.162 mM gentam.	113 ± 16	2
	0.323 mM gentam.	295 ± 94	3
	0.801 mM DEH	335 ± 17	1
	4.190 mM glucos.	110 ± 8	1
Neutral§	None	111 ± 13	4
	0.216 mM gentam.	128 ± 10	1
	0.323 mM gentam.	114 ± 19	1
	0.431 mM gentam.	125 ± 8	1
	0.801 mM DEH	106 ± 9	1
	4.190 mM glucos.	113 ± 7	1

* Gentam., gentamicin; DEH, bis(beta-diéthylaminoethylether)hexestrol; glucos., 2-D-glucosamine.

† ± SD. Each sample was analyzed six times. The total number of determinations was thus six times the number of independent experiments.

‡ Liposomes made as described in Materials and Methods (cholesterol:phosphatidylcholine:sphingomyelin:phosphatidylinositol; 5.5:4:5:2).

§ Liposomes containing no negatively charged phospholipids (cholesterol:phosphatidylcholine:sphingomyelin; 5.5:4:7).

compared to control liposomes at both 298°K and 310°K. Liposomes incubated in the presence of gentamicin or glucosamine showed a small but non-significant decrease in fluorescence polarization as compared to controls. The results obtained at the other temperatures investigated (from 288°K to 328°K) were similar.

Light scattering spectroscopy studies

Light scattering spectroscopy was used to examine the effect of gentamicin and DEH on the apparent diameter of liposomes. Sonicated liposomes were used since these showed a smaller, more reproducible and less heterogeneous diameter than unsonicated liposomes (data not shown). As shown in Table 2, addition of gentamicin resulted in a significant increase of the apparent size of negatively-charged liposomes at a drug concentration of 0.323 mM. The magnitude of this effect, however, was very variable both between different liposome preparations and within the same preparation (data not shown). DEH also induced a significant increase of the size of the negative liposomes, which was much more reproducible than for gentamicin. 2-D-glucosamine had no effect up to molar concentrations exceeding 10-fold the concentration of gentamicin causing a significant effect. Table 2 also shows that omission of phosphatidylinositol in the liposomes abolished the effect of both gentamicin and DEH, indicating that negative charges in the bilayer were essential in this respect for either compound.

^{31}P NMR spectra at fixed temperature

The influence of gentamicin and DEH on liposome apparent size was further examined by analysis of the ^{31}P-NMR spectra at a fixed temperature of 298°K. Figure 2A (panel A1) shows that control, sonicated liposomes display only a narrow peak (isotropic signal), indicating that the phosphate heads are highly mobile. Addition of either gentamicin (panel A3) or DEH (panel A4), at an equinormal concentration, resulted in a dual signal in which a broad component with a low field shoulder and a high field peak were superimposed on the isotropic signal. 2-D-glucosamine (panel A2), at a molar concentration 13-fold higher than gentamicin, showed no effect. Figure 2B shows the ^{31}P-NMR spectra obtained at 298°K with unsonicated liposomes. In the absence of drug (panel B1), it showed both a narrow isotropic signal and a broad signal with its maximum at high field (panel B1). With addition of increasing amounts of gentamicin (panels B2 to B5), the narrow signal decreased and the broad asymetric signal, with a low field shoulder and a high field peak characteristic of large bilayer structures, steadily increased.

Morphological studies

Figure 3 shows the morphological appearance of replicas prepared from freeze-etched negatively-charged liposomes. Compared to controls (panel 3A), it clearly appears that the increase of liposome apparent size in the presence of gentamicin (panel 3B) mostly resulted from an aggregation of the vesicles. Morphometric analysis confirmed that the size of most individual profiles remained close to that of control liposomes (mean diameter: 25 nm), and only few vesicles displaying a diameter exceeding 100 nm. In striking contrast, panel 3C shows that, at an equinormal concentration, DEH induced the formation of large vesicles with a diameter of approx.

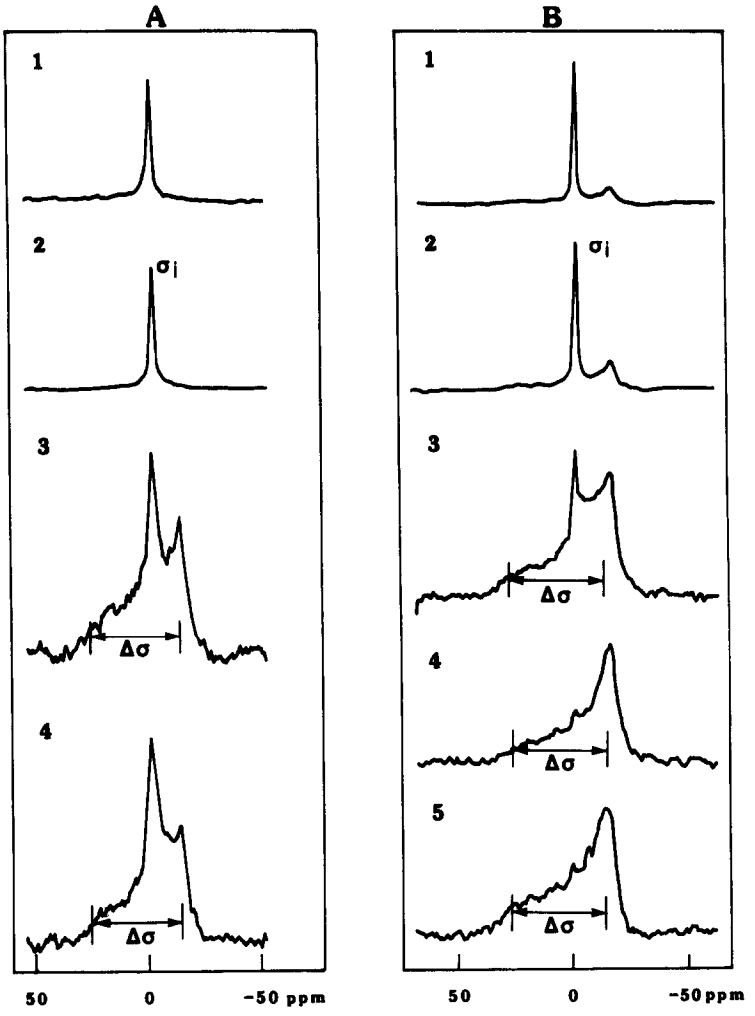


Fig. 2. ^{31}P-NMR spectra of sonicated (A) or unsonicated liposomes (B). The composition and concentration of the liposomes were as in Fig. 1. Recording was made at 298°K and at a frequency of 101.3 MHz. A1 and B1, control liposomes; A2, A3, A4, sonicated liposomes incubated 1 hr at 37° with 4.2 mM glucosamine, 0.32 mM gentamicin or 0.8 mM DEH, respectively; B2–B5, unsonicated liposomes incubated 1 hr at 37° with increasing concentrations of gentamicin (0.11, 0.22, 0.32 and 0.43 mM). σ_i at 0 ppm is the chemical shift of phosphorus atoms undergoing isotropic motion. To allow comparison among the different spectra, the intensity of the maximal signal is set on an identical value.

170 nm, while the original, small-sized liposomes were much less numerous. No morphometric analysis of DEH-treated liposomes was performed in view of the magnitude and consistency of the effect. In addition to its effect on liposome size, DEH also altered the morphology of the liposome membrane, which displayed evenly distributed, round-shaped small inclusions. Such inclusions were never seen in control or in gentamicin-treated liposomes.

^{31}P *NMR lipid polymorphism studies*

Since morphological studies clearly indicated that DEH induced fusion of the liposomes, and perhaps also a change in their membrane organization, the influence of DEH on lipid polymorphism was examined using ^{31}P-NMR spectroscopy. For this study, we used liposomes made of dioleoylphosphatidylethanolamine and dielaidoylphosphatidylethanolamine (1:1, w/w), since these allow a better characterization of specific phospholipid macromolecular organizations such as hexagonal phases. Figure 4 shows the change of ^{31}P-NMR spectra of these liposomes when the temperature was increased from 268°K up to 314°K. Control liposomes displayed a marked temperature transition between 302°K and 306°K. The spectrum at low temperature showed the characteristic shape of an axially symmetric shielding tensor. At high temperature, the chemical shift anisotropy was reduced twofold and displayed a reverse asymmetry. Liposomes exposed to gentamicin (or to 2-D-glucosamine; data not shown) behaved as controls. In striking contrast, addition of DEH caused, already at a temperature as low as 286°K, the appearance of a narrow signal at σ_i, intermediate between the two maxima detected on the spectra of control liposomes recorded at the low and the high

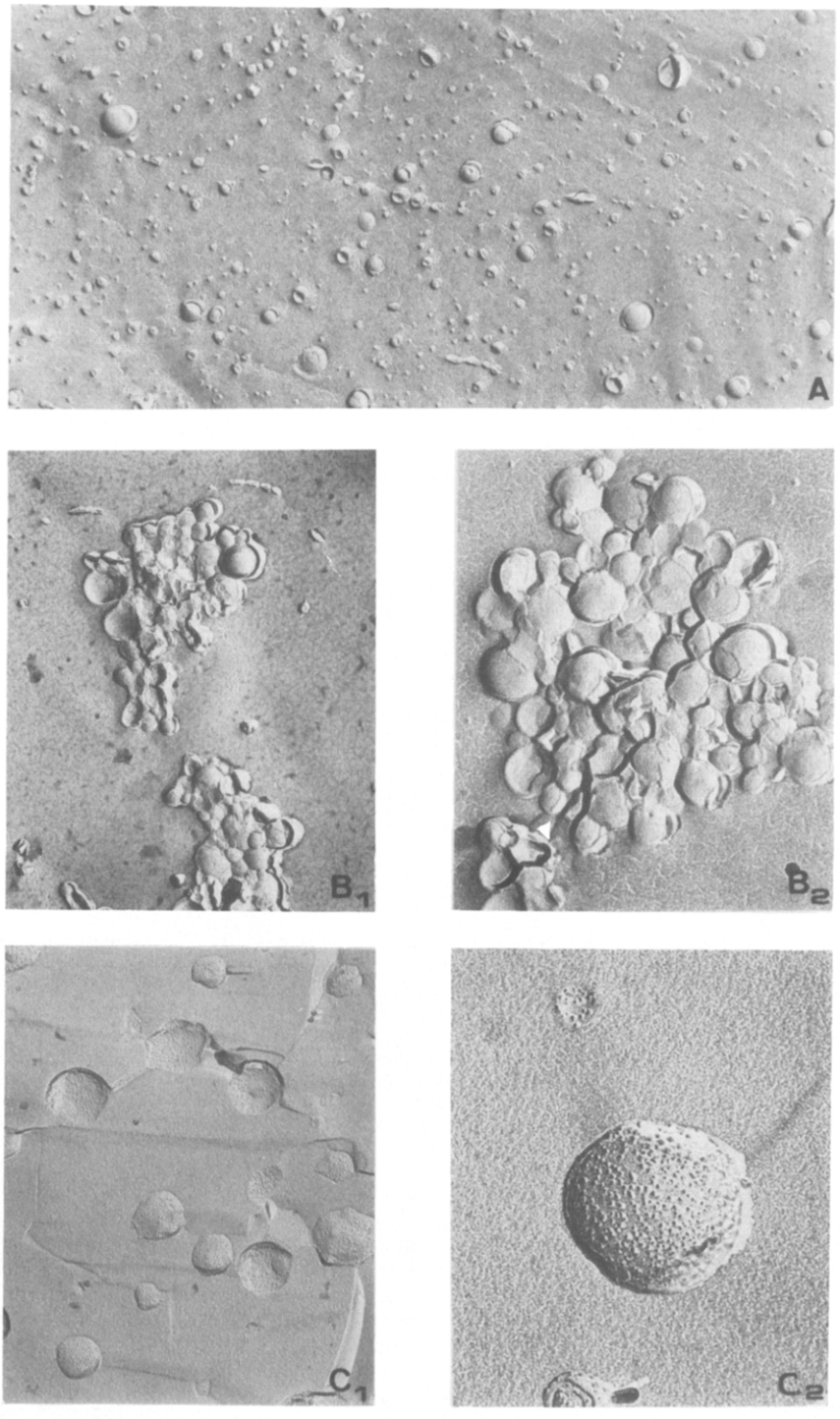


Fig. 3. Morphological appearance of negatively charged, sonicated liposomes after freeze-etching. The liposomes were of the same composition and concentration as that used in Fig. 1; A, control liposomes; B, C: liposomes incubated with equinormal concentrations of gentamicin (B, 0.32 mM) or DEH (C, 0.8 mM). Magnifications: A, B1, C1, 37,500×; B2, C2, 85,000×.

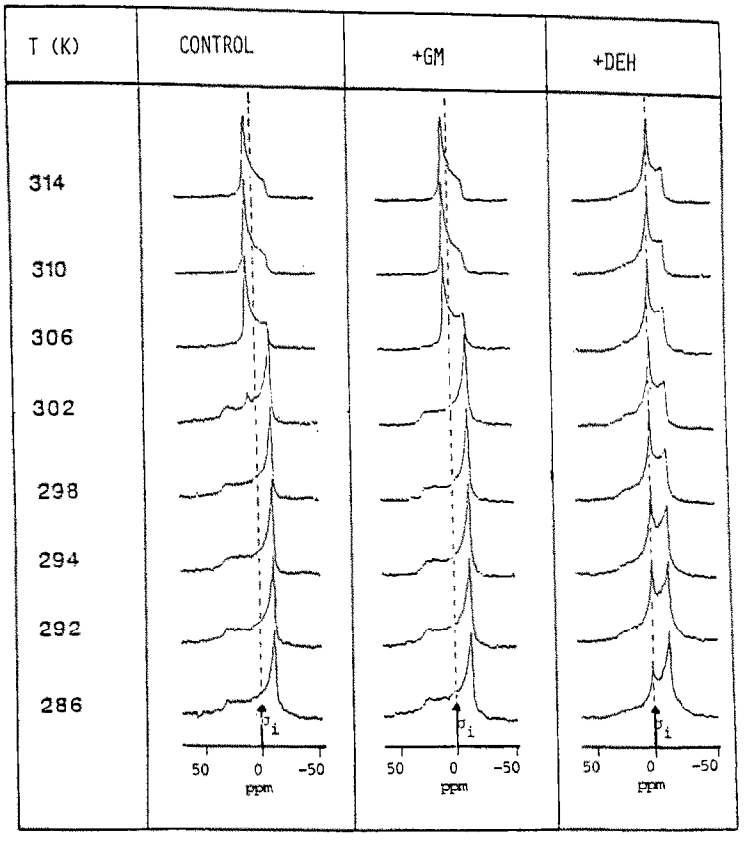


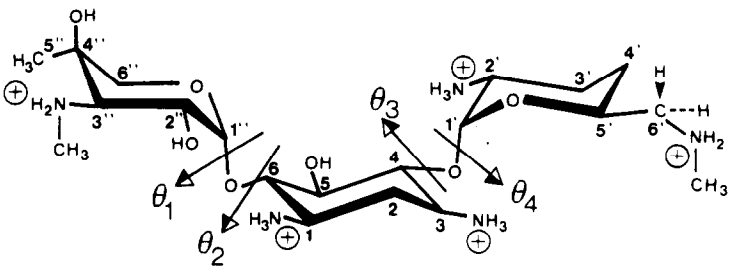
Fig. 4. ^{31}P-NMR spectra of liposomes made of dioleoylphosphatidylethanolamine and dielaidoylphosphatidylethanolamine (1:1) at increasing temperature. Gentamicin (GM) or DEH were added at a final ratio drug:lipids 1:3. The 0 ppm value is set at σ_i corresponding to the isotropic shift value.

temperature, respectively. When the temperature was raised, the magnitude of this signal increased in parallel with the decrease of the original broad signal.

Conformational studies

(a) *Isolated molecules.* Molecules were analyzed under their fully protonated forms, since all previous biochemical and biophysical studies have been made at pH 5.4, i.e. at a pH value below the pK_a of the amino-groups of either drug. This pH is also that probably prevailing in lysosomes [20, 21]. The structural formulae of gentamicin and DEH and the torsional angles of importance in this analysis are shown in Fig. 5. The results of the conformational analysis of the isolated gentamicin molecule at a lipid–water interface and stereoviews of the most probable (over 99%) conformer have been published by us previously [28, 32] but appear again here (Fig. 6) for comparison, since the mode of representation used in the present paper (full-size atomic volumes) is different from that used earlier. The main characteristics of this conformer are given in Table 3. For DEH, the systematic analysis performed on the torsional angles θ_1, θ_2, θ_3, θ_4 and θ_5 and the simplex minimization procedure applied while orienting the conformers at a lipid–water interface, yielded only one highly probable conformer (99.5%). This conformer is shown in Fig. 6 and its main characteristics are given in Table 3. In contrast to gentamicin, which adopted a largely open crescent shape, with its concavity oriented towards the hydrophobic phase, DEH oriented at a lipid–water interface displayed an almost closed hairpin shape with its concavity directed towards the hydrophilic phase. The two amino groups of the molecule were close to each other at one extremity of the molecule and the phenyl groups exposed at the other. Consequently, the distance between the hydrophilic and hydrophobic gravity centers was very large and the molecule span across the interface. The aminogroups could therefore completely be located in the hydrophilic zone, whereas the phenyl groups entirely stayed in the hydrophobic zone. When a similar analysis was repeated for DEH by simulating a totally hydrophobic environment, DEH adopted a completely different conformation, in which the molecule was fully extended (data not shown). No conformer was calculated for gentamicin in an hydrophobic environment since this molecule is not lipid-soluble.

(b) *Molecules inserted in phosphatidylinositol monolayers.* A maximum of four molecules of phosphatidylinositol could be assembled around either gentamicin or DEH. Table 4 shows the calculated energies of interactions and energies of transfer (hydrophobic and hydrophilic) between each drug and the surrounding phosphatidylinositol molecules.



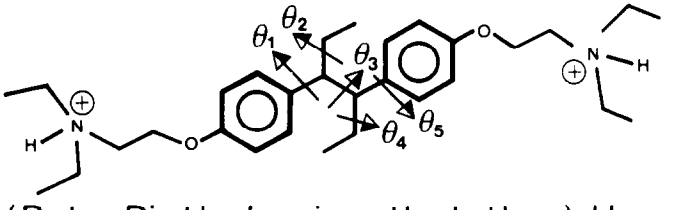


Fig. 5. Structural formulae of gentamicin and DEH. Gentamicin, as most clinically-used aminoglycosides (except streptomycin and neomycin), is made of a 2-deoxystreptamine moiety substituted by 2 amino-sugars. In accordance with a widely accepted convention [59], the sugar moiety substituting the 6-hydroxyl group of the 2-deoxystreptamine (3-deoxy-3(methylamino-4-*C*-methyl-beta-L-arabinopyranose), situated to the left in the figure) is referred to as the "sugar, whereas that substituting the 4-hydroxyl group of the 2-deoxystreptamine (2,6-diamino-2,3,4,6-tetradeoxy-alpha-D-*erythro*-hexopyranose), situated to the right in the figure), as the ' sugar, respectively. The component of gentamicin shown in this figure, and in Figs 6 and 7, is the C1a (components C1 and C2 carry an additional methyl group in N6' or C6', respectively). The systematic name of DEH is 2,2'-(1,2-diethyl-1,2-ethanediyl)bis(4,1-phenyleneoxy)bis (*N*,*N*-diethyl-ethanamine). The figure also shows the torsional angles (gentamicin, θ_1 to θ_4; DEH, θ_1 to θ_5) which were systematically varied for the conformational analysis. The values of these angles in the most probable conformers are given in Table 3.

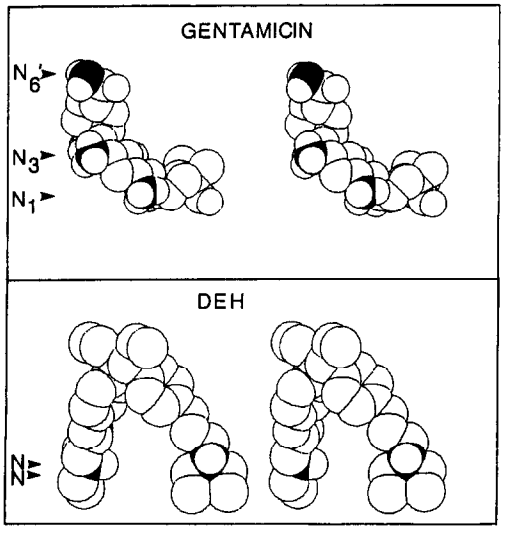


Fig. 6. Space-filling stereoviews of the most probable conformers of gentamicin and DEH at a water–lipid interface (the lipid phase being above). The positions of the ionizable aminogroups are indicated by the arrows on the left of the figure pointing to the N atoms (in black) visible in the figure (see Fig. 5 for numbering conventions).

Compared to gentamicin, DEH showed a much higher energy of interaction, and the energy balance was much in favour of hydrophobic interactions. Figure 7 illustrates the most probable location and orientation of phosphatidylinositol molecules assembled around gentamicin or DEH, and shows how each drug interacts very differently with them. For gentamicin, the point of lowest energy was obtained when the hydrophilic/hydrophobic interface of the phosphatidylinositol monolayer was misshaped, as shown in Fig. 7. Thus, the four phosphatidylinositol molecules could not be located on the same plane, and one (numbered 1 in Fig. 7) was placed as if it were attracted towards the aqueous phase, whereas a second (numbered 4 in Fig. 7) was displaced towards the hydrophobic phase. This position was imposed by the gentamicin molecule, which, in spite of its open crescent shape, remained therefore completely at or close to the hydrohilic/hydrophobic interface. In particular, the N'6 amino-group of gentamicin could establish a close contact with the phosphate head of a phosphatidylinositol molecule. These positions of the phosphatidylinositol molecules around gentamicin are more plausible than the ones that we proposed earlier [28, 32], in which this N'6 amino function of genta-

Table 3. Main characteristics of gentamicin and DEH conformers after simplex minimization procedure and orientation at a stimulated lipid–water interface

	Torsional angles (degrees)*					Distance between hydrophobic and hydrophilic centres (Å)
	θ_1	θ_2	θ_3	θ_4	θ_5	
Gentamicin	223	154	217	119		0.60
DEH	300	60	0	180	300	2.38

* See Fig. 5 for the numbering of the torsional angles.

Table 4. Calculated interaction and transfer energies, and molecular area of the complexes formed between DEH or gentamicin and phosphatidylinositol

	Energy of interaction*	Transfer energy*		Molecular area† Å^2
		Hydrophobic	Hydrophilic	
Gentamicin	−44.7‡	214.43	214.02	52
DEH	−112.8	258.66	57.39	60

* kJ/mol.

† Area of the drug molecule projected on the lipid–water interface.

‡ The value shown here is larger than that reported earlier (−35.2 kJ; see Ref. 32), because of the improvements introduced in the analysis of the complexes.

micin penetrated into the hydrophobic domain of the monolayer. The mis-shaping of the interface also favoured the interactions of the other amino-groups of gentamicin with the surrounding phosphatidylinositol molecules. The mode of insertion of DEH was in striking contrast to that of gentamicin. First, only a minor perturbation of the hydrophilic/hydrophobic interface of the monolayer was apparent. Second, the DEH molecule inserted itself amidst the phosphatidylinositol molecules, almost in parallel to

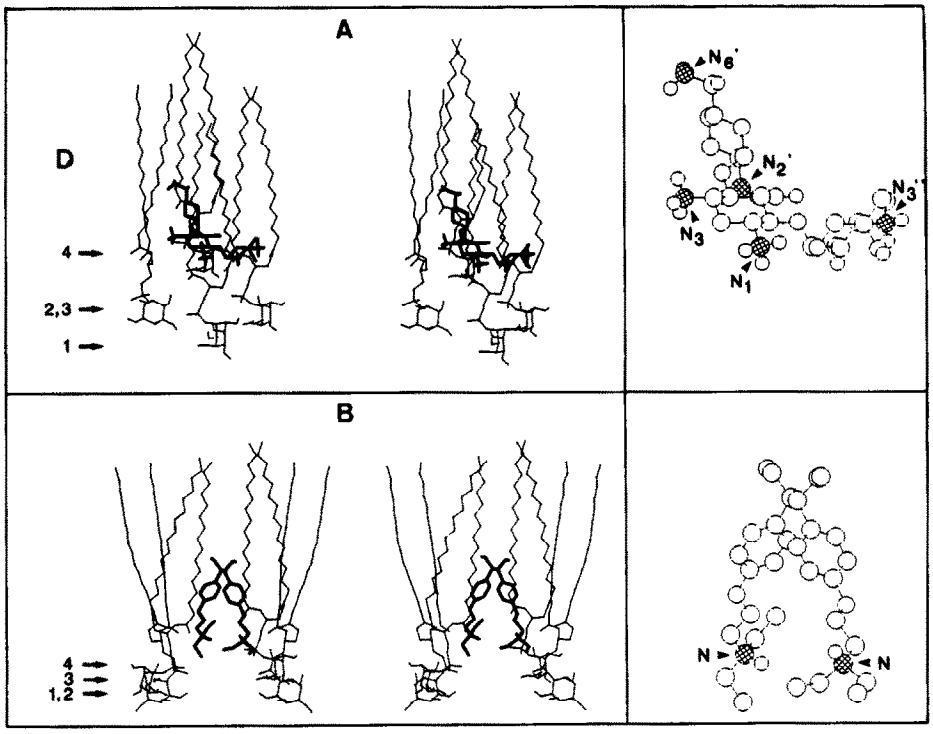


Fig. 7. Skeleton stereoviews of the mode of assembly of gentamicin (A) or DEH (B) and phosphatidylinositol. In order to facilitate the orientation, the ball representation of the drugs with the same conformation as they adopt in the complex, are displayed in the right panels. The solid arrows on the left panel refer to the positions of the inositol moieties of the four phosphatidylinositol molecules surrounding the drug.

the fatty acid chains. Thus, the two aminogroups remained located below the hydrophobic–hydrophilic interface, where they probably established close electrostatic interactions with the phospho groups, whereas the phenyl groups and the diethylethanediyl moiety joining them were fully inserted into the hydrophobic part of the bilayer, establishing close contacts with entire segments (C4 to C8) of the adjacent aliphatic chains.

DISCUSSION

Both gentamicin and DEH accumulate in lysosomes where they reach concentrations in the millimolar range [3, 10, 11] and are expected to be fully protonated, since the lysosomal pH is around 5–6 [20, 21]. They could therefore bind to the membranes of these organelles and/or to the cellular membranes carried into lysosomes by autophagy [36], since most cellular membranes [19], including those of lysosomes [37], contain negatively-charged phospholipids. In the present study, we have used a somewhat simplified model of membrane containing only phosphatidylinositol, as a negatively charged phospholipid, and phosphatidylcholine, sphingomyelin and cholesterol as "structural" phospholipids. Yet the use of membrane layers of composition similar to or simpler than that used here already have allowed it to be shown that (a) both aminoglycosides and cationic amphiphiles displace Ca^{2+} from negatively-charged membranes [13, 18, 38, 39]; (b) that cationic amphiphiles interact with membranes by both hydrophobic and hydrophilic forces [40, 41]; (c) that binding of gentamicin to a membrane requires negative charges [15] and primarily results from the electrostatic potential prevailing in the aqueous layer adjacent to that membrane [42]. Together with the data summarized above, the experimental observations and the results of the calculations reported in this paper show that both gentamicin and DEH interact with the hydrophilic domain of the negatively-charged membranes, but that only DEH can influence the properties of the hydrophobic domain.

The results of the ^{31}P NMR spectroscopy show that both gentamicin and DEH cause a significant restriction in the movements of the phosphate heads of the phospholipids. The data of our conformational analysis support this interpretation, the calculated positions of the amino groups in both DEH and gentamicin being such that they can establish close contact with the negatively charged phospho groups of phosphatidylinositol. Mobility could be impaired by bridging several phosphatidylinositol molecules if complexes of an order higher than 1:1 (drug: phosphatidylinositol molar ratio) are formed, as shown by our conformational analysis. The binding of gentamicin to liposomes is indeed cooperative with respect to the phosphatidylinositol content [32]. Moreover the drug concentration necessary to obtain charge reversal of negatively charged phospholipid vesicles decreases when their content in phosphatidylinositol or phosphatidylserine increased [42], which suggests that the binding of gentamicin is favoured by the closeness of the negative charges on the membrane surface. Bridging several negative charges also explain the aggregation of the liposomes caused by gentamicin (see below for a discussion of the effect of DEH).

In contrast to their common effect on the mobility of the phosphate heads, DEH and gentamicin very differently affect specific properties of the hydrophobic domain of the phospholipids. Thus, in accordance with the lack of detection of a boundary potential within the membrane in liposomes exposed to gentamicin [42], the results of the fluorescence polarization studies made with 1,6-diphenyl-1,3,5-hexatriene, which is a totally hydrophobic fluorochrome, show that gentamicin binding to the liposomes is not associated with a significant interaction with the hydrophobic domain of the bilayer. Compared to our previous proposal [32], our new conformational model indeed shows that, because of the deformation of the interface it induces, gentamicin can remain entirely within the hydrophilic region in spite of its open-crescent shape. Conversely, DEH causes a significant decrease of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene embedded in the liposomes, reflecting an increased mobility of the hydrocarbon chains [26, 43]. This effect can be understood on the basis of the conformational model proposed for DEH interacting with a phosphatidylinositol monolayer. Furthermore, the ^{31}P-NMR spectroscopic examination of dioleoyl- and dielaidoylphosphatidylethanolamine liposomes (Fig. 4) shows that DEH interferes with the change in lipid reorganization that occurs in these membranes upon warming, causing the appearance of a new type of lipid organization indicated by a narrow peak at σ_i characteristic of a phase where isotropic motion occurs. This isotropic signal could be due to the formation of inverted micelles within the membrane [44]. By contrast, the spectra of control liposomes or of liposomes after addition of gentamicin are similar, and the modification of the shape of the spectrum seen upon increase of the temperature is indicative of a normal transition from a bilayer to an hexagonal organization of the lipids [44]. The marked fusion of liposomes induced by DEH further points out to a specific interaction of this drug with the hydrophobic domain of the membrane, since a profound reorganization of fatty acid chains is probably necessary to induce bilayer fusion [45–47]. The roundly shaped intramembranous particles seen after addition of DEH (Fig. 3C) could represent inverted micelles, the formation of which has indeed been associated with membrane fusion phenomena [48–50]. Intramembranous inclusions similar to those shown here, have also been observed in the limiting membrane of lamellated inclusions appearing in the retinal tissue of animals treated *in vivo* by cationic amphiphiles, after freeze-fracture examination [51].

In addition to its interaction with the hydrophobic domain, however, DEH also needs to establish electrostatic interactions with the bilayer to cause fusion of the vesicles, since no increase in apparent size is seen when DEH is added to neutral liposomes (Table 2). A general conclusion of our work could therefore be that an agent is fusogenic only if it can both attach to the surface of the bilayer (polar domain) and insert itself into the hydrophobic domain. Thus, the exclusive interaction of gentamicin with the polar

domain of the negatively charged bilayers would prevent it from causing fusion, whereas its polycationic character would favour vesicle aggregation when its concentration reaches a critical threshold. Whether by fusion or aggregation, however, large bilayer structures will be formed, which explains the similar changes seen in the ^{31}P NMR spectra upon addition of either gentamicin or DEH (Fig. 2). A similar appearance or a large bilayer signal has been observed in multilamellar phosphatidylserine vesicles exposed to gentamicin or Mg^{2+} [42]. The formation of aggregates upon exposure of liposomes to gentamicin probably also accounts for the apparent increase in size observed by quasi-elastic light scattering spectroscopy. Discrepancies between the absolute values of the mean liposome diameters recorded by light scattering spectroscopy and by morphometry of the freeze-etched replicas, respectively, and the apparently very large variability from one measurement to another may be accounted for by the fact that the former type of measurement is strongly influenced by the presence of a small number of large particles.

The differences observed between the interactions between phospholipid bilayers and gentamicin or DEH, taken as a typical aminoglycoside and a typical cationic amphiphile, respectively, may result in a different organization of the phospholipids accumulated *in vivo* upon treatment with either drug. This could lead to different toxicities. Buckheim *et al.* [51] have observed that the polar lipids accumulating in retinal cells after treatment with chlorphentermine, a cationic amphiphile, often display specific structural organizations consistent with those observed *in vitro* in the present study, namely a lattice-like pattern and intramembranous inclusions which have not been reported in gentamicin-induced phospholipidosis [10, 52] nor in inherited phospholipidoses (see Ref. 53 for review). A major difference between the phospholipidosis induced by the aminoglycosides and that caused by cationic amphiphiles is that the former is quickly associated with cytotoxicity whereas the latter results in a huge accumulation of intralysosomal myeloid bodies without apparent sign of cell death [57]. Further comparative biochemical and biophysical studies of the lipids accumulated upon exposure to either drug may help to unravel and assess better the meaning of these morphological and functional differences.

We reported earlier [16] that gentamicin and DEH are equipotent inhibitors of lysosomal phospholipase A1, assayed *in vitro* towards phosphatidylcholine included in negatively-charged liposomes, when these drugs are added at equinormal concentrations. Although cationic amphiphiles could also act on the enzymes [41, 58], it is tempting to speculate that interactions with the polar domains of the phospholipid layers, and in particular charge neutralization is the critical determinant in this inhibition. Yet, hydrophilic compounds with only three positive charges, such as streptomycin, are much less potent inhibitors than gentamicin, or DEH, even at equinormal concentrations [32]. We therefore suggest that the hydrophobic interactions established between DEH and the hydrocarbon chains are probably important for confering enough stability to the complex between this drug and the negatively charged bilayers in spite of its low number of cationic functions. A similar conclusion was reached by Lüllmann *et al.* [18] concerning the capacity of cationic amphiphiles to displace Ca^{2+} from negatively charged monolayers, compared to aminoglycosides [39]. We do not known, however, whether this conclusion can be extended to monoaminated amphiphilic drugs such as chlorphentermine or chloropromazine, which also induce phospholipidosis. Moreover, experiments in which the intralysosomal concentration of an aminoglycoside and an amphiphilic cationic drug would be correlated with the extent of the phospholipidosis they cause, will also need to be performed before the present *in vitro* observations can be taken as correctly describing the situation prevailing *in vivo*.

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