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INTERACTION OF STREPTOMYCIN AND STREPTOMYCYLAMINE DERIVATIVES WITH NEGATIVELY CHARGED LIPID LAYERS

CORRELATION BETWEEN BINDING, CONFORMATION OF COMPLEXES AND INHIBITION OF LYSOSOMAL PHOSPHOLIPASE ACTIVITIES

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Abstract—Aminoglycoside antibiotics induce a lysosomal phospholipidosis in kidney proximal tubules after conventional therapy in animals and man. We have previously demonstrated that these drugs bind to negatively charged phospholipid bilayers at acid pH and inhibit the activity of lysosomal acid phospholipases *in vitro* and *in vivo*. A combined biochemical and conformational study [Brasseur *et al.*, *Biochem. Pharmac.* **33**, 629 (1984)] showed major and consistent differences between 6 aminoglycosides in current clinical use with respect to the stability of the complexes they form with phosphatidylinositol, their inhibitory potency towards the activity of lysosomal phospholipases and their current toxicity ranking (e.g. gentamicin > amikacin > streptomycin). In the present study we have extended this approach to experimental derivatives of streptomycin. The derivatives examined were: dihydrostreptomycin, dideguanyldihydrostreptomycin, streptomycylamine, dideguanylstreptomycylamine, *N*-butyl- and *N*-benzyl-dideguanylstreptomycylamine. These compounds were examined for (i) their binding to negatively charged liposomes, measured by gel permeation on Sepharose 4B; (ii) their interactions with phosphatidylinositol assessed by semi-empirical conformational analysis and (iii) their inhibitory effect on the activities of lysosomal phospholipases towards phosphatidylcholine present in negatively charged liposomes. Streptomycin and gentamicin were also used as reference compounds with low and high affinity (and inhibitory potency), respectively.

Our observations can be summarized as follows: (i) the replacement of the aldehyde in the streptose ring by a methylamino group strikingly changes the conformation of the molecule, allowing a better interaction with phosphatidylinositol. Thus, streptomycylamine binds much more tightly to phospholipid bilayers and shows a higher inhibitory potency towards phospholipase activity, as compared to streptomycin. The conformational analysis shows, however, that this effect is only partially due to the additional cationic charge carried by streptomycylamine. Other modifications of the streptomycin molecule, such as the replacement of the guanidinium groups by aminogroups or the addition of hydrophobic moieties (butyl or benzyl groups) to the streptose do not markedly further strengthen the interactions of the molecule with phosphatidylinositol. (ii) Even though some derivatives (e.g. dideguanylstreptomycylamine) bind as tightly to phospholipids as gentamicin, they remain much less inhibitory towards lysosomal phospholipases. All streptomycin derivatives extend parallel to the fatty acid chains and across the hydrophobic–hydrophilic interface, whereas gentamicin is oriented largely parallel to this interface and above the plane of the phospho groups. We therefore conclude that the position of an aminoglycoside in the bilayer is as crucial as the tightness of the binding (more related to the number of cationic groups) in causing inhibition of phospholipase activities.

Aminoglycoside antibiotics are both oto- and nephrotoxic [1]. Whereas the exact mechanism of either toxicity remains unknown, convergent data show that aminoglycosides specifically interact with negatively charged phospholipids, especially at acid pH [2–4]. This binding is likely to change the organization and properties of phospholipid bilayers and thus interfere with a number of biological processes. We also have shown that the binding of aminoglycosides to bilayers results in an inhibition of the activities of lysosomal phospholipases towards both negatively charged and neutral phospholipids ([7] and manuscript in preparation). The relevance of the latter observations to the *in vivo* situation stems from the fact that aminoglycosides induce a conspicuous impairment of phospholipid catabolism in the lysosomes of kidney [4, 8, 9]. Lysosomes display an acid pH [10]. Several animal and clinical studies have also shown that aminoglycosides accumulate in the lysosomes *in vivo* ([6, 9] and references therein).

Finally, there appears to be a fair correlation between the inhibition of lysosomal phospholipid catabolism *in vivo* (phospholipidosis) and the nephrotoxic potential of aminoglycosides (compare, for example, the data in refs 4, 9 and 11 concerning the phospholipidosis to those related to toxicity in refs 12–15; see also refs 16, 17). Moreover, recent data show that phospholipidosis precedes and is thereafter associated with tissue necrosis and regeneration [18, 19]. Aminoglycoside-induced phospholipidosis is not restricted to kidney tissue, but also affects other cell types provided these are exposed to a sufficiently high drug concentration, e.g. the eye conjonctiva and eye pigmented epithelium after local injection *in vivo* [20, 21], or cultured fibroblasts *in vitro* [22].

In a previous study [23], we have applied the method of conformational analysis to examine the interactions of several aminoglycosides used in clinical practice with phosphatidylinositol. We observed a relationship between the transfer energy involved in these interactions, the degree of binding and the ability of each drug to inhibit phospholipase activities. In all respects, gentamicin was the most, and streptomycin the least able to interact with phospholipid layers. Strikingly, streptomycin is almost non-nephrotoxic *in vivo* [24]. We now have used closely related streptomycin derivatives to explore in more detail the relationship between the binding of the drug to phospholipid layers, the interaction with phosphatidylinositol as examined by conformational analysis, and the inhibition of lysosomal phospholipase activity. Our approach has been refined to assess not only the most probable conformation of the aminoglycosides at a hydrophobic–hydrophilic interface, but also to obtain probability maps of the various conformations.

MATERIALS AND METHODS

Aminoglycosides and other chemicals. Streptomycin was obtained from Laboratorium Wolfs (Antwerpen, Belgium) and complied with the Belgian Pharmacopea. The various derivatives, the structural formulae of which are given in Fig. 1, were synthetized as previously described [25]. Their purity (>90%) was checked by thin-layer chromatography. Gentamicin was supplied by Schering Corporation U.S.A. (Kenilworth, NJ). Only streptomycin, dihydrostreptomycin and gentamicin have significant antibacterial activity (data not shown). All aminoglycosides were either supplied or prepared as the sulfate salts and the pH was carefully adjusted to approx. 5.4 before use. Lipids were purchased from Lipid Product (Nr Redhill, U.K.) or Sigma Chem. Co. (St Louis, MO); radiolabelled phosphatidylcholine was obtained from Amersham International (Amersham, Bucks., U.K.); Sepharose 4B from Pharmacia AB (Uppsala, Sweden). The other products were of analytical grade.

Binding and phospholipase inhibition studies. These methods have been fully described in previous publications [4, 7]. In brief, we used sonicated liposomes (phosphatidylcholine, sphingomyelin, phosphatidylinositol and cholesterol; molar ratio 4:4:3:5.5) and binding was measured by gel filtration through Sepharose 4B (in 4 mM acetate buffer pH 5.4). Bound aminoglycoside is the fraction of drug eluting with the liposomes peak and is expressed in nmole of aminoglycoside/μmole of phospholipid. Assay of drugs was made with fluorescamin, or by a microbiological method for streptomycin.

The activities of the lysosomal phospholipase A_1 (EC 3.1.1.32) and of the sum of phospholipase A_2 (EC 3.1.1.4) + β-lysophospholipase (lysophosphatidylcholine 2-deacylase; EC 3.1.1.–) were determined with liposomes containing 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine as a substrate. The enzyme source was a soluble extract from highly purified liver lysosomes. The IC_{50}s (concentrations causing 50% inhibition) of aminoglycosides were determined by adding increasing concentrations of the drug to the assay mixture.

Conformational analysis. As described previously [23], a stepwise computation approach was used to predict the configuration of mixed monolayers of aminoglycoside and phosphatidylinositol. The values used for valence angles, bond lengths, atomic charges and torsional potentials were those in current use for conformational analysis [26]. All cationic groups are given a positive charge, since the analysis is intended to describe the phospholipid/aminoglycoside interactions thought to occur in lysosomes *in vivo* [4], i.e. at a pH of 5.5 or below [10]. To obtain the conformations of the isolated molecules and their orientation at a hydrophobic–hydrophilic interface [27–29], the following parameters were calculated (these details were not given in ref. 23):

1. The London–Van der Waals energy of interaction between all pairs of non-mutually bound atoms. Buckingham's pairwise atom-atom interactions functions have been used [30, 31]

$$E^{VdW} = \sum_{ij} [A_{ij} \exp(-B_{ij} r_{ij}) - C_{ij} r_{ij}^{-6}]$$

were $j = 1, 2, \ldots$ are non-mutually bound atoms, r_{ij} their distances from each other and A_{ij}, B_{ij} and C_{ij} are coefficients assigned to atoms pairs. The values of these coefficients have been reported [32, 33]. In order to compensate for the decrease of the function, E^{VdW} has been set to 100kcal/mole at $r_{ij} < 1$ Å.

2. The generalized Keesom–Van der Waals interaction or electrostatic interaction between atomic point charges.

$$E^{cb} = 332 \left(\sum_{ij} \frac{e_i e_j}{r_{ij} \varepsilon_{ij}} \right)$$

where e_i and e_j are expressed in electron charge units, r_{ij} in Å, and ε_{ij} as the dielectric constant. The values of the atomic point charges are similar to the values used for polypeptides [26].

3. The potential energy related to the rotation of covalent bonds (torsional angles). This rotation around the C—C, C—O or C—N bonds was calculated by the equation:

$$E^{tor} = \frac{U_{ij}}{2} (1 + \cos \phi_{ij})$$

where U_{ij} corresponds to the energy barrier in the eclipsed conformation during the rotation of the angle and ϕ_{ij} to the torsional angle. The values used for periodicity, sometimes referred to as multiplicity values, were those of Hopfinger [26].

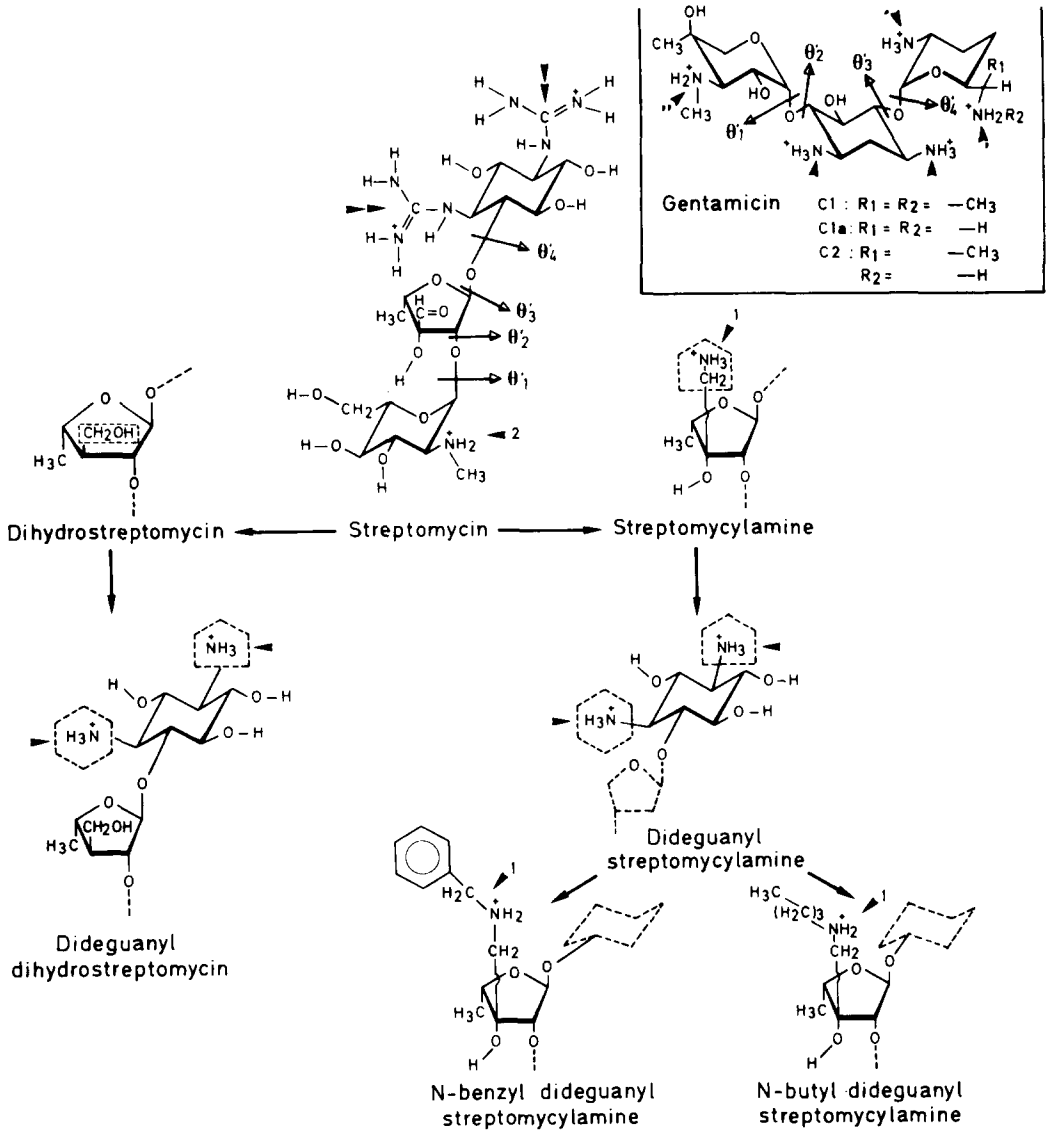


Fig. 1. Structural formulae of streptomycin and of the derivatives studied. Streptomycin systematic is *O*-2deoxy-2(methylamino)-α-L-glucopyranosyl-(1 → 2)-*O*-5-deoxy-3-C-formyl-α-L-lyxofuranosyl-(1 → 4)-*N*-*N*′-bis(aminoiminomethyl)-D-streptamine. The trivial names for the lyxofuranosyl and streptamine moieties, as substituted in streptomycin, are streptose and streptidine. The double arrows (▶▶) indicate the carbon of the guanidinium groups in streptidine (streptomycin, streptomycylamine); the single arrows (▶) indicate the aminogroup replacing the guanidium group in streptamine (dideguanylstreptomycylamine, *N*-butyl- or *N*-benzyl-dideguanylstreptomycylamine); the single arrows marked 1 (1▶) indicate the aminogroup present on the lyxofuranosyl moiety (streptomycylamine, dideguanylstreptomycylamine, *N*-butyl- or *N*-benzyl-dideguanylstreptomycylamine); the single arrows marked 2 (2▶) indicate the secondary amine in the glucopyranosyl moiety (all compounds). The inset shows the structural formula of gentamicin. Commercially available gentamicin is a mixture of C_1, C_{1a} and C_2 components (approx. molar ratio: 30:25:45). The systematic name of gentamicin C_{1a} is: *O*-3-deoxy-3-(methylamino)-4-*C*-methyl-β-L-arabinopyranosyl-(1 → 6)-*O*-2,6 diamino-2, 3, 4, 6 tetradeoxy-α-D-erythrohexopyranosyl-(1 → 4)-2-deoxy-D-streptamine. The arrows (▶) indicate the amino-groups on the 2-deoxystreptamine, the arrows marked ′ ('▶) indicate the aminogroups on the erythrohexopyranosyl and that marked ″ ("▶) the secondary amine on the arabinopyranosyl moiety.

4. The transfer energy of each part of the molecule. The values of the transfer energies used are similar to those determined experimentally by numerous authors and summarized elsewhere [34]. In the calculation procedure, six changes of 60° each were first imposed to each of n torsional angles, yielding 6^n conformers. The conformational energy was calculated for each of these conformers. The most probable conformations were identified as those yielding the lowest internal energy. Such a selection is based

on the statistical weight (Boltzmann) of all individual configurations. After systematic analysis, conformations selected for their lowest internal energy were submitted to a simplex minimization procedure [35].

A simulated air–water interface was defined as follows. To one atom of the molecule was attributed a plane, above which the dielectric constant is equal to 3. The lowest atom below this plane was used as a reference for a second parallel plane, the dielectric constant of which has a value of 30. Between these 2 planes, the dielectric constant was assumed to increase linearly along the z axis perpendicular to the simulated interface. The molecule was finally oriented with the line joining the hydrophilic 2nd hydrophobic gravity centers perpendicular to the interface. The hydrophilic gravity centre ($\vec{C}_w$) is defined by the following equation:

$$\vec{C}_w = \sum_{i=1}^{n} [E^+ \text{ transfer}_i \, \vec{r}_i] \Big/ \sum_{i=1}^{n} E^+ \text{ transfer}_i$$

in which $\vec{r}_i$ are the coordinates of the i atom. The hydrophobic gravity centre located in the hydrocarbon domain ($\vec{C}_{HC}$) is defined by the same equation, except that the negative transfer energies are taken into account. The interface position ($\vec{I}$) is defined by the equation:

$$\frac{\sum_{i=1}^{n} E^+ \text{ transfer}_i}{\vec{C}_w - \vec{I}} = \frac{\sum_{j=1}^{m} E^- \text{ transfer}_j}{\vec{C}_{HC} - \vec{I}}$$

The molecular structures and the numbering of the torsional angles, in the streptomycin derivatives are illustrated in Fig. 1.

To construct maps of energies and probabilities, a rotation of 10° was imposed to two adjacent rotational angles. In this condition, 36 structures were calculated for each rotational angle. Therefore, the combination of the rotation around the two angles gave 1296 structures. For each structure, the conformational energy was calculated. This energy is the sum of the Van der Waals interaction, the electrostatic interaction and the torsional potential. From E_i, the energy of the ith case above the minimal value of all cases, a Boltzmann probability (P_i) was calculated. Two maps were therefore drawn, showing the energy surface and the probability surface respectively. The most probable structure corresponds to a maximum in the probability map and to a flat or hollow area in the energy surface map.

The construction of a monolayer and the insertion of an aminoglycoside molecule was then made as previously described by stepwise approach [23, 36] starting from two molecules and repeating the procedure up to a total of 4 phosphatidylinositol per aminoglycoside, at which point the mean molecular area was found to reach a stable value. Calculations were made on CDC-CYBER 170 computer coupled to a calcomp 1051 drawing table (Computing Centre of Free University of Brussels). The drawing program (PLUTO) was kindly provided by Dr A. Englert [37].

RESULTS

Experimental measurements

Table 1 shows the binding and the inhibitory properties of streptomycin and its derivatives, and of gentamicin for comparison purposes. As compared to streptomycin, the reduction of the aldehyde group (dihydrostreptomycin) alone or combined with the replacement of the guanidium radicals by primary amines (dideguanyldihydrostreptomycin) is without much influence on the inhibitory properties.

Table 1. Correlation between binding of aminoglycosides to liposomes and inhibition of phospholipases

Aminoglycoside	Binding* (nmole of aminoglycoside per μmole of lipid phosphorus)	Inhibition of enzymatic activity† (aminoglycoside concentration causing 50% inhibition in μM)	
		Phospholipase A_1	Phospholipase A_2 + β-lysophospholipase
Streptomycin	2.2	409 ± 51	335 ± 65
Dihydrostreptomycin	n.d.	354 ± 3	308 ± 5
Dideguanyl dihydrostreptomycin	4.4	427 ± 8	405 ± 26
Streptomycylamine	9.8	254 ± 10	221 ± 15
Dideguanyl streptomycylamine	n.d.	253 ± 4	233 ± 6
N-butyldideguanyl streptomycylamine	11.0	232 ± 2	207 ± 5
N-benzyldideguanyl streptomycylamine	11.8	196 ± 5	180 ± 15
Gentamicin‡	11.4	112 ± 19	116 ± 13

* Aminoglycosides were mixed with liposomes (0.1 μmole drug for 10 μmole lipid phosphorus) and the molar ratio drug/phospholipid was determined in the liposome-peak after gel permeation on Sepharose 4B. Thus, a figure of around 10 indicates the binding of all drug present on the sample (figures may be slightly higher than 10 since the aminoglycoside and phospholipid profiles may not be entirely superposable). Data shown are the mean of the 2 experiments.

† Activity of phospholipase A_1 is measured by the release of labelled lysophosphatidylcholine; the release of labelled fatty acid results from the activity of phospholipase A_2 and lysophospholipase. Data shown are mean ± S.D. of at least 3 independent experiments (Ref. 7 for experimental details).

‡ Data from refs. 7 and 23.

n.d.: not determined.

Table 2. Main characteristics of the isolated aminoglycoside molecules at a simulated hydrophobic/hydrophilic interface

	Torsional angles*				Distance between hydrophobic and hydrophilic centers (Å)	Hydrophilic Hydrophobic balance (kcal/mole) / (kcal/mole)
	θ'_1	θ'_2	θ'_3	θ'_4		
Streptomycin	226	21	207	260	0.89	48.4/68.4
Streptomycylamine	251	32	323	64	0.91	49.3/71.6
Dideguanyl streptomycylamine	290	18	300	83	0.48	46.3/61.7
N-butyl-dideguanyl streptomycylamine	268	8	300	70	1.53	56.0/60.7
N-benzyl-dideguanyl streptomycylamine	287	14	306	87	1.73	57.7/60.7
Gentamicin	223	154	217	119	0.60	51.3/51.2

* In degrees; precision is ±5°; see Fig. 1 for the definition of the angles.

Conversely, addition of an aminogroup on the streptose moiety (streptomycylamine) considerably enhances the inhibition of enzymatic activities. Streptomycylamine also binds considerably more to the lipid vesicles, and almost no free drug was detected after gel filtration. Addition of a hydrophobic moiety (benzyl or butyl radical) only slightly increases the inhibitory potency of the derivatives. In comparison, gentamicin is considerably more inhibitory, although the molar ratio aminoglycoside: phospholipid is the same as that observed for streptomycylamine and its derivatives.

Conformational analysis

The conformational analysis of the phosphatidylinositol has been run previously [23]. Table 2 shows the values of the torsional angles located between the cycles (θ'_1, θ'_2, θ'_3, θ'_4; see Fig. 1) of streptomycin and its derivatives after the simplex minimization procedure and orientation of the molecules at the simulated air–water interface. Corresponding values for gentamicin [23] are given for comparison.

Table 2 also gives the distance between the hydrophobic and hydrophilic gravity centres, and the hydrophobic–hydrophilic balance of each molecule. Energy and probability maps were calculated to explain the strikingly different values of the angle θ'_4 between streptomycin and streptomycylamine (Fig. 2). Whereas the energy maps are similar for angles θ'_3 and θ'_4 in either compound, the probability maps appear strikingly different. For streptomycin, the region of maximal probability corresponds to 210° ± 30° for θ'_3 and 240° ± 30° for θ'_4; in the case of streptomycylamine, this region corresponds to 340° ± 30° for θ'_3 and 50° ± 30° for θ'_4. This difference is not only due to electrostatic repulsion originating from the amino residue added to the streptomycin molecule, but also to steric hindrance since it is still partly observed when the charges are omitted in our calculations.

Figure 3 shows the most probable conformations of streptomycin, along with streptomycylamine and its derivatives viewed along the 3 perpendicular axes. Streptomycylamine and its derivatives adopt a more extended conformation than streptomycin. This might have important implication on the packing of the phosphatidylinositol molecules around the aminoglycoside.

For each aminoglycoside, the most probable conformers were inserted into the simulated phosphatidylinositol monolayer but only the assembling modes corresponding to the minimal energy were retained. The structures shown in Fig. 4 correspond to a probability greater than 95%. The interaction energy E_{in} (sum of the Van der Waals interaction energy, E_{VdW}, and the electrostatic interaction, E_{elec}), the relative position and the mean area occupied by each drug molecule are listed in Table 3. Only two cationic groups in streptomycin and in all derivatives establish strong electrostatic interactions with the negatively charged phosphogroups of the phosphatidylinositol. These groups are either the two guanidium groups or the two aminogroups carried by the streptamine moiety (streptomycin or streptomycylamine and dideguanylstreptomycylamine respectively). The difference noted above in the value of the torsional angles between streptomycin and streptomycylamine plays an essential role in the orientation of the aminoglycoside and in the packing of the phospholipids around the drug. The more extended conformation of streptomycylamine indeed allows contact between the guanidium groups and the phospho groups. The additional cationic group by itself, however, is unlikely to play a major role in the electrostatic binding, because it appears too far from the phospho groups. The addition of a butyl moiety to the amino group located on the lyxofuranosyl moiety of dideguanylstreptomycylamine allows a closer association between the aminoglycoside and the hydrophobic chains of the phospholipids. Yet, the hydrophobic energy of interaction remains low. Rather surprisingly, a benzyl moiety does not much increase either the hydrophobic energy of interaction. As shown in Fig. 4, however, it causes a tilting of the fatty acid chains of the phosphatidylinositol relatively to the interface. We have not yet explored how far this

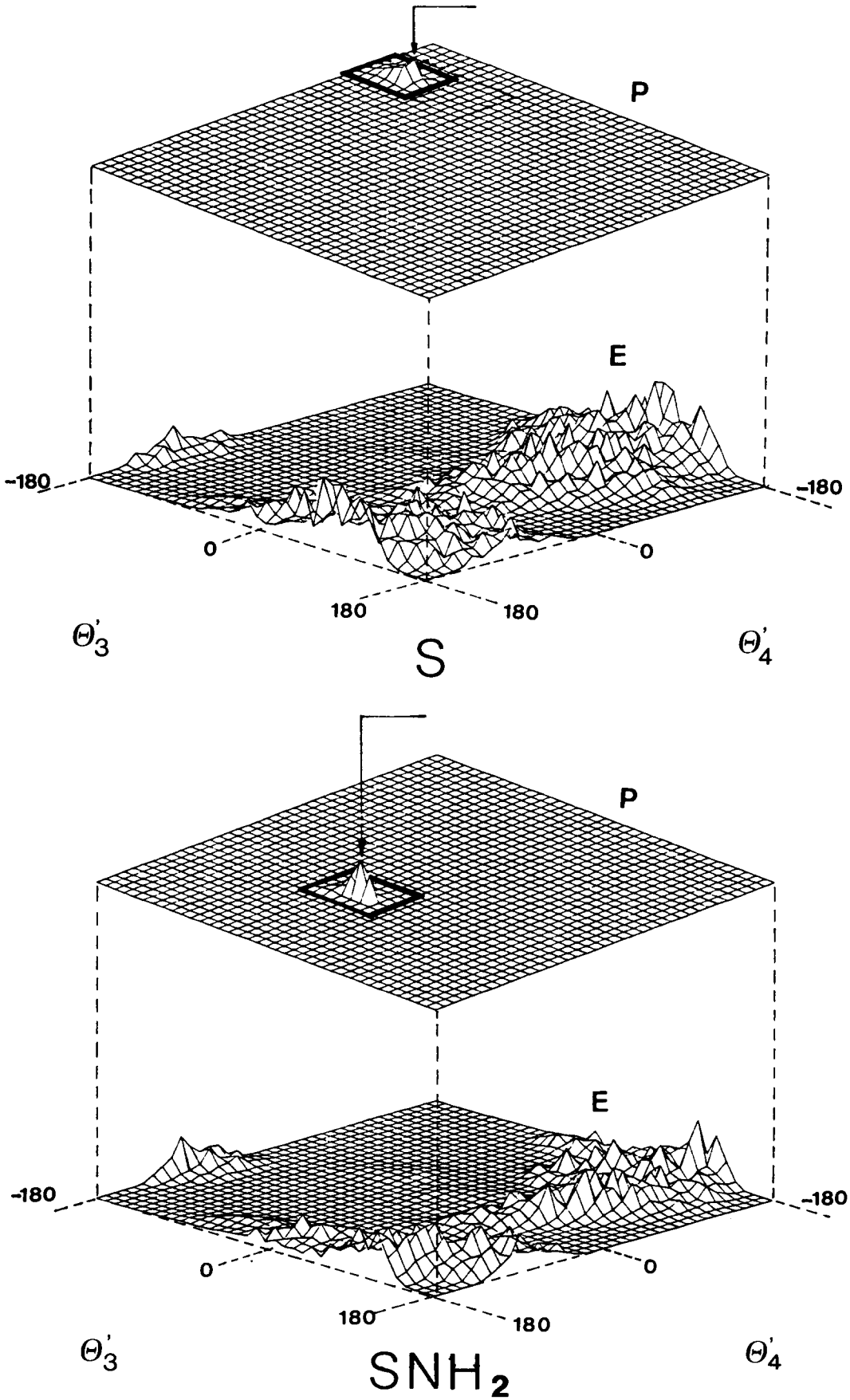
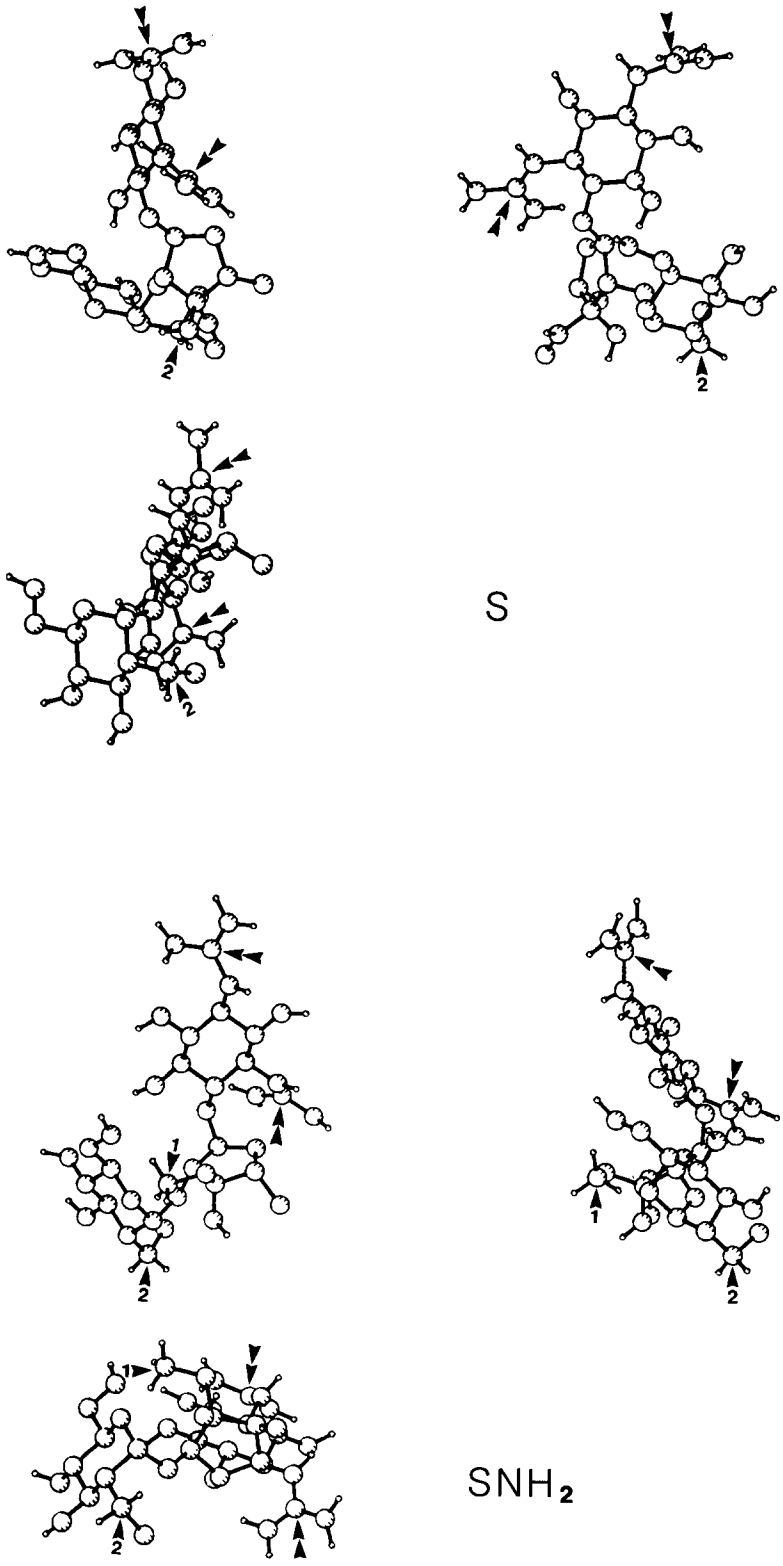


Fig. 2. Energy (E) and probability (P) maps of streptomycin (S) and streptomycylamine (SNH_2). The peaks on the upper part of the diagram point to the values of θ'_3 and θ'_4 corresponding to the most probable conformation. The flat or hollow area in the lower part of the diagram corresponds to the lowest energy conformations.



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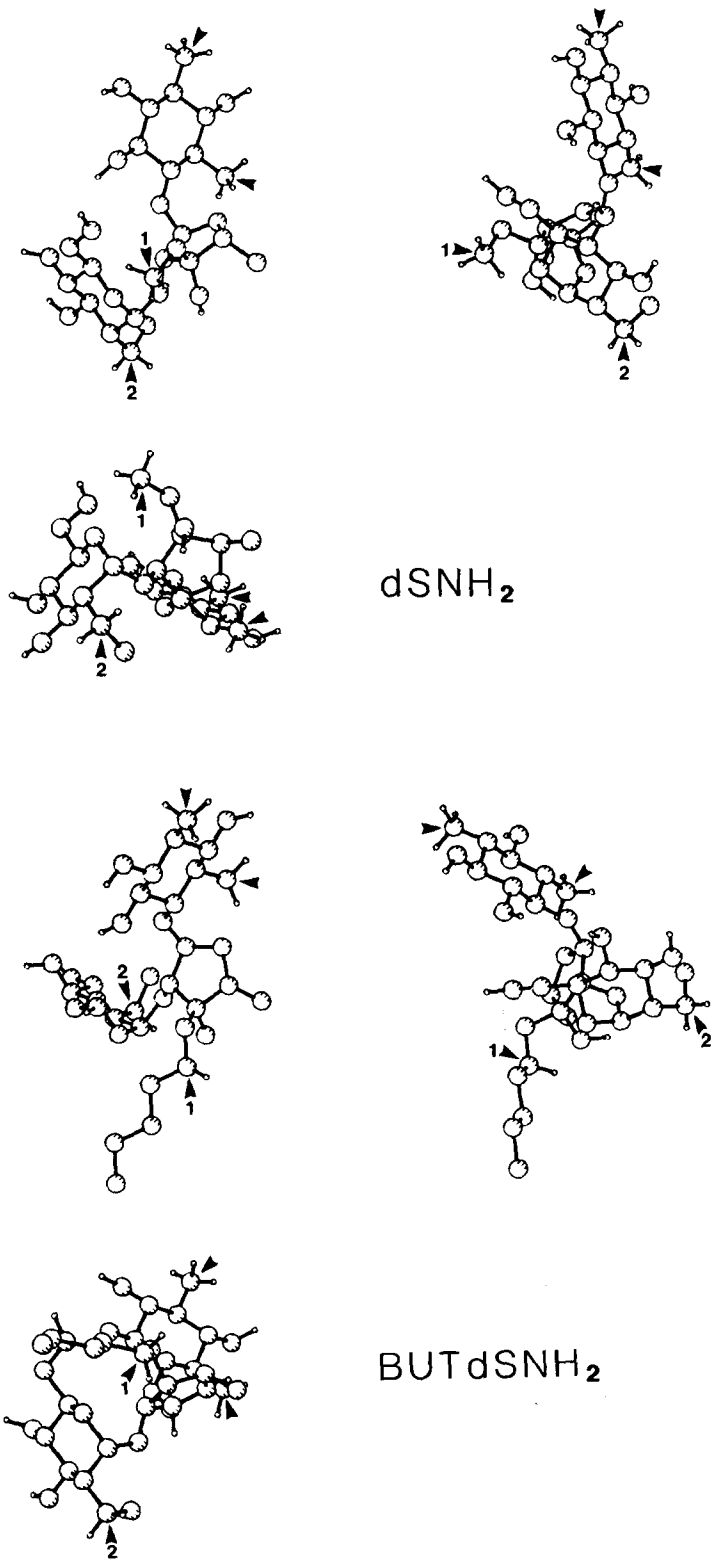

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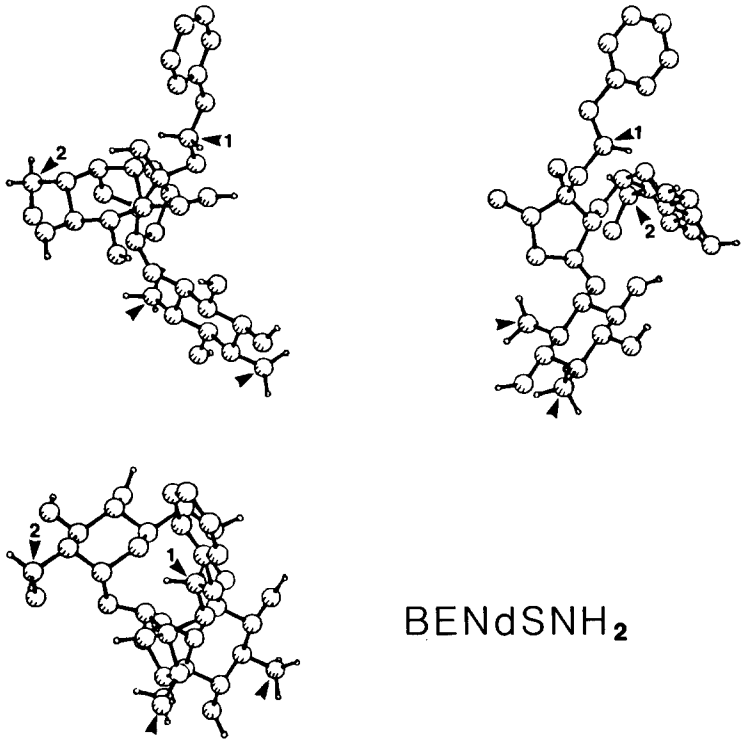

Fig. 3. Conformation of streptomycin, streptomycylamine and parent compounds after minimization procedure. Each molecule is viewed along three perpendicular axes. The symbols (▶, ▶▶, ^{1}▶, ^{2}▶) are defined in Fig. 1. Key: S, streptomycin; SNH_2, streptomycylamine; $dSNH_2$, dideguanylstreptomycylamine; $BUTdSNH_2$, *N*-butyl-dideguanylstreptomycylamine; $BENdSNH_2$, *N*-benzyldideguanylstreptomycylamine.

disorganization extends in the phosphatidylinositol monolayer.

A major difference between streptomycin (and all its derivatives) and gentamicin is the degree and the mode of insertion of the molecule into the monolayer. Streptomycin extends largely parallel to the backbone of the fatty acyl chains (i.e. vertically in Fig. 4) and is partly inserted between the phospho groups and between the inositol moieties. Free contact between adjacent inositol moieties is therefore impaired. Conversely, gentamicin is disposed almost perpendicular to the fatty acyl chains (i.e. almost horizontally in Fig. 4) and is entirely above the plane of the phospho groups. Thus the inositol moieties

Table 3. Main characteristics of the interaction of streptomycin, streptomycylamine derivatives and gentamicin with a phosphatidylinositol monolayer

	Molecular area (Å^2)	Position with respect to phosphatidyl monolayer inositol†	Energy of interaction (kcal/mole) Hydrophobic	Hydrophilic
Streptomycin	41.3	—P↓*	−0.5	−3.0
Streptomycylamine	35.0	—P↓*	−1.2	−7.3
Dideguanyl streptomycylamine	35.0	—P↓*	−0.6	−8.4
N-butyl-dideguanyl streptomycylamine	41.2	—P↓*	−1.9	−9.1
N-benzyl-dideguanyl streptomycylamine	50.2	—P↓*	−1.8	−9.8
Gentamicin‡	52.0	↓P—*	−0.5	−7.9

† The P indicates the phosphorus group and the * the inositol moiety. The arrow indicates the position of the aminoglycoside atom which is the most oriented towards the aqueous phase.

‡ Data partially from Ref. 23.

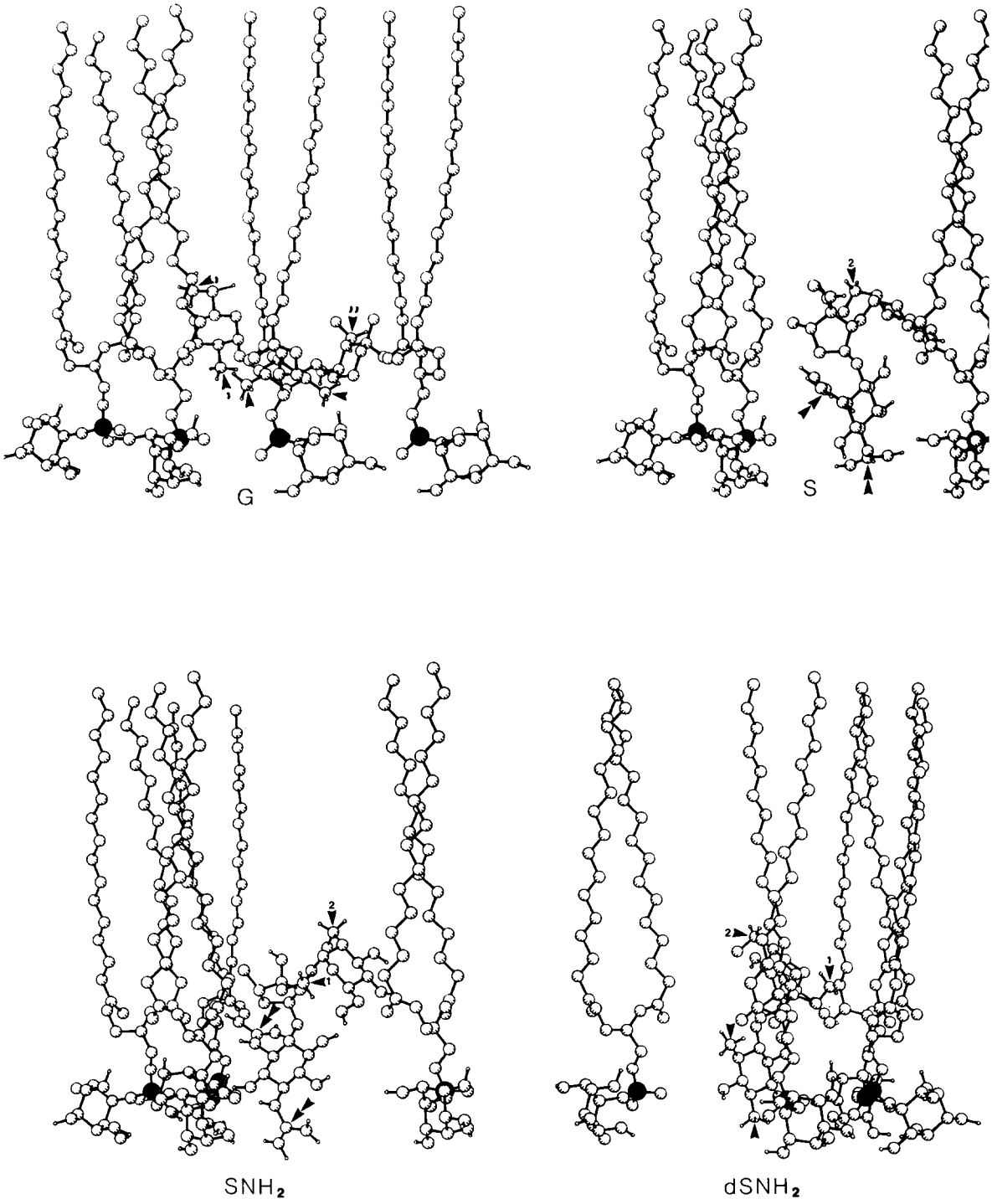

Fig. 4 (*continued*).

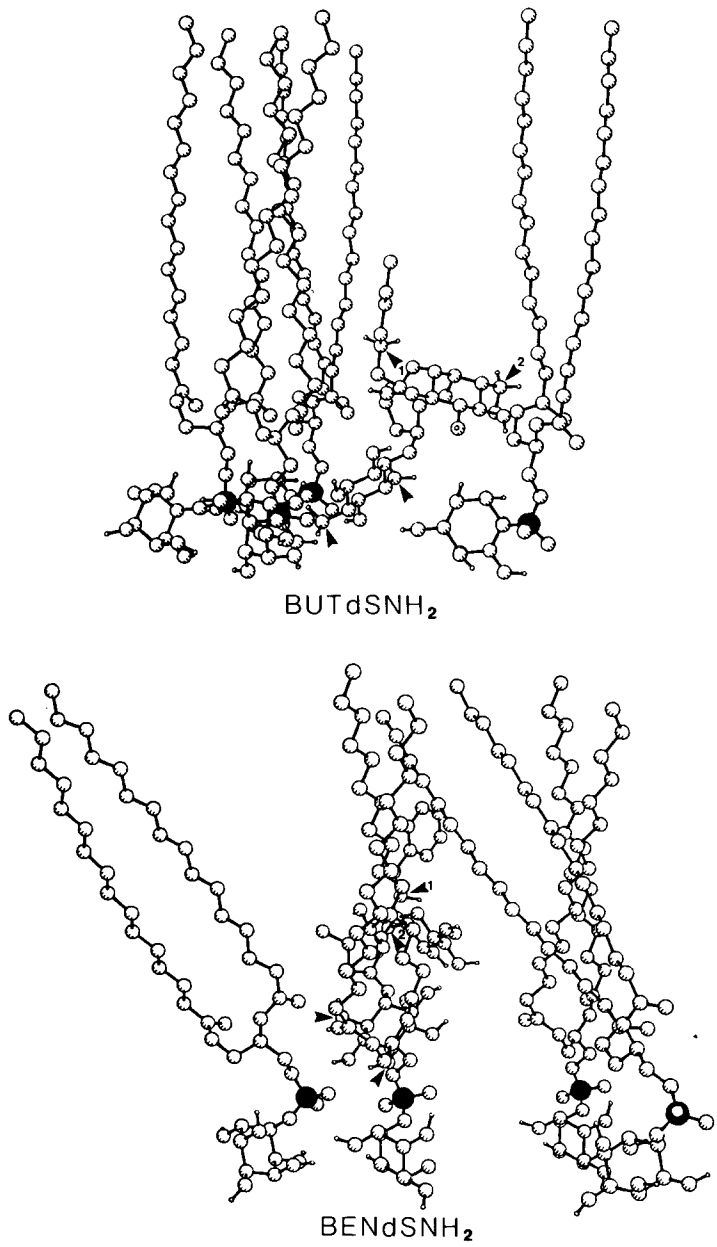

Fig. 4. Conformation of drug–phosphatidylinositol mixed monolayers. The black circles indicate the phosphorus atoms; the cationic groups of the aminoglycosides are identified by arrows as described in the legend to Fig. 1. Abbreviations are defined in Fig. 3. G is gentamicin. The component of gentamicin shown is the C_{1a}; no major difference was noted between this component and the other two (C_1, C_2).

are allowed an almost unhindered contact between themselves. This position of gentamicin also allows more cationic groups to come close to the phospho groups (3 out of 5, at positions N_1, N_3 and N'_2, respectively).

DISCUSSION

The present paper extends our previous analysis [23] of the molecular parameters responsible for the binding of aminoglycosides to negatively charged lipid layers at acid pH and for the subsequent impairment of lysosomal phospholipase activities. As stated in the introduction and discussed in earlier publications [4, 7], we consider that this approach may be relevant to the nephrotoxicity of aminoglycosides. Indeed, *in vivo* investigations have shown that lysosomal phospholipidosis (associated with an impairment of the activity of phospholipase Al) is an early and significant alteration of proximal tubular cells after exposure to those drugs [9, 11, 16, 17, 19] (see also refs 6, 8). Because lysosomes display an acid

pH, probably equal to or lower than 5.5 *in vivo* [10] and contain acidic phospholipids [38], we have used these conditions in our *in vitro* experiments. The concentrations of drugs are also in the range or lower than those achieved in lysosomes *in vivo* [9]. We also have assumed for our conformational analysis that all basic groups of the drug molecules are protonated. The lowest pK_a value reported for gentamicin is 5.5, with all other groups displaying higher pK_as [39]. We have not obtained physico-chemical determinations of the pK_as of the various cationic groups in streptomycin and streptomycylamine derivatives. The guanidinium groups are certainly protonated at pH 5.5. By analogy with the data reported for tobramycin [40], neomycin [41], and kanamycin [42], we may, however, reasonably assume that the pK_a of the aminogroup in the glucopyranosyl moiety of streptomycin and that of the aminogroup added to the lyxofuranosyl moiety (for obtaining streptomycylamine and its derivatives) will be higher than 7. The aminogroups of the streptamine (in the dideguanyl derivatives) should range between 5.7 and 8.4 [40–42]. In this regard, our conclusions may differ from those of other studies which analyse the properties or the conformations of aminoglycosides at higher pH.

A first important conclusion is that binding of aminoglycosides to phospholipids is not directly related to antibacterial activity. Indeed, both streptomycin and gentamicin are active antibiotics, whereas all other derivatives studied here have no or little antibacterial activity. At first glance, a key factor in the binding of aminoglycosides appears to be the number of positively charged groups in the molecule, since we demonstrate a major difference between streptomycin (3 cationic groups) and streptomycylamine (4 cationic groups). The conformational analysis, however, suggests that in either case only 2 cationic groups strongly interact with the phospho groups. Thus the more prominent binding of streptomycylamine has to be explained by change in the shape of the molecule rather than by additional electrostatic binding. We found earlier that amikacin—a structurally different aminoglycoside also with 4 aminogroups—has an energy of interaction of only −4.9 kcal/mol [23]. Thus, a second conclusion is that the relative position of the cationic groups is certainly as important as their number, a point which may have often been overlooked (see e.g. ref. 6). Conversely, the nature of the cationic groups (guanidium vs primary amine, or primary amine vs secondary amine) seems largely unimportant, at least in the examples investigated here.

As shown in Tables 1 and 3, consistent differences were found for the binding of different aminoglycosides, as measured experimentally, and for their energy of interaction calculated by the conformational analysis. This parallelism thus lends further support to the relevance of the latter approach. At first glance, it is surprising that *N*-butyl- or *N*-benzyldideguanylstreptomycylamine do not show a higher energy of interaction since butyl or benzyl groups are likely to increase the hydrophobicity of the molecule. The conformational analysis, however, shows that the presence of hydrophobic moieties at the position considered (i.e. attached to lyxofuranosyl moiety) does not markedly increase the depth of insertion of the aminoglycoside into the bilayer. Hydrophobic moieties attached to other places in the molecule may, however, have more marked effects. At this time, we have no simple explanation for the disturbing effect of benzyl moiety on organization of the fatty acid chains, nor do we have data concerning its relevance to drug toxicity. Other aminoglycosides showing a similar effect are under study [43].

When streptomycin derivatives are compared, there is a clear relationship between binding and inhibitory potency towards phospholipases. However, examination of gentamicin behaviour makes it clear that binding (and energy of interaction) are not the only factors determining the inhibition of enzymatic activity. Indeed, streptomycylamine or *N*-benzyldideguanylstreptomycylamine bind tightly to liposomes but remain considerably less inhibitory than gentamicin (Table 1) or even than kanamycin A or amikacin (two aminoglycoside with 4 aminogroups) [7, 23]. A major difference between all streptomycin derivatives studied here and gentamicin is also their shallower insertion into the bilayer (Table 3). The position of the two types of drugs is also largely different. Amikacin, which binds less than gentamicin and is also less inhibitory, displays a more vertical position than streptomycin even though it is somewhat more deeply inserted [23]. Thus, a third important conclusion of our studies is that both a tight binding and a deep insertion of the aminoglycoside into the lipid layer are probably critical for the inhibition of phospholipid hydrolysis. Conceivably, one might decrease the inhibitory potency of a given aminoglycoside towards lysosomal phospholipases either by reducing its energy of interaction with (and therefore the binding to) negatively charged phospholipids, or by preventing its deep insertion into the bilayer. The second approach may be more realistic to obtain less nephrotoxic aminoglycosides for clinical use, because it could be achieved without modifying the number of aminogroups. Some of the latter are indeed critically important for antibacterial activity [44, 45].

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