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ACCESSIBILITY OF AMINOGLYCOSIDES, ISOLATED AND IN INTERACTION WITH PHOSPHATIDYLINOSITOL, TO WATER

A CONFORMATIONAL ANALYSIS USING THE CONCEPT OF MOLECULAR HYDROPHOBICITY POTENTIAL

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Abstract—The mode of interaction between aminoglycosides and negatively charged phospholipids plays a critical role in the inhibition of lysosomal phospholipases induced by these antibiotics and therefore in their nephrotoxicity. Previous works suggested that accessibility of the drug interacting with phospholipids to water could be crucial in this respect. We have used the concept of molecular hydrophobicity potential described by Brasseur [*J Med Chem* **266**: 16120–16127, 1991] to visualize the hydrophobic and hydrophilic envelopes around aminoglycosides assembled with phosphatidylinositol molecules, and to obtain a three-dimensional representation of the complex formed. Using a series of different aminoglycosides, we showed that molecules with a lower inhibitory potential (gentamicin B, amikacin and isepamicin) are surrounded by both hydrophobic and hydrophilic envelopes whereas aminoglycosides which are more inhibitory are enveloped primarily by either hydrophilic (kanamycin A or B) or hydrophobic (gentamicin C_{1a}) envelopes. This approach, which is here for the first time applied to the study of drug–lipid complexes, could help in the better understanding of the molecular mechanism of lysosomal phospholipase inhibition induced by aminoglycosides.

Aminoglycoside antibiotics often remain essential for the treatment of severe infections caused by Gram (−) bacteria because of their excellent chemotherapeutic properties. A severe limiting factor in their use, however, is their oto- and nephrotoxicity [1–3]. With respect to nephrotoxicity, aminoglycosides have been shown to concentrate in the lysosomes of kidney proximal tubular cells [4, 5], to inhibit the activities of lysosomal phospholipases A and C, and of sphingomyelinase [6—8], and to induce a lysosomal phospholipidosis [6, 7]. The latter metabolic alteration is probably a first, critical event leading to tubular necrosis and nephrotoxicity (see reviews in Refs 9, 10), even though the mechanism(s) relating phospholipidosis to cell necrosis remain uncertain. The inhibition of lysosomal phospholipases by aminoglycosides can be reproduced *in vitro* [6, 11]. Using liposomes, we actually showed that the activity of lysosomal phospholipase A_1 towards phospholidylcholine is critically dependent on the presence of negatively charged phospholipids in the bilayer [12–14]. As aminoglycosides bind to acidic phospholipids [6, 14–16], we postulated that the presence of these drugs impairs the activity of phospholipase A_1 because it decreases the amount of negative charges available to the enzyme [13]. In this context, the inhibition of the enzyme should primarily be correlated to the amount of drug bound and to the tightness of its binding to the membrane. However, a series of observations challenge this simple hypothesis. First, specific aminoglycoside derivatives, such as dideguanylstreptomycylamine, bind as tightly to negatively charged bilayers as gentamicin but are considerably less inhibitory towards lysosomal phospholipases [17]. Second, a recently described new aminoglycoside, 6″-deoxy-6″-isopropylthio-kanamycin B, was shown to be much more inhibitory than its parent compound, 6″-deoxy-kanamycin B, although the energies of interaction of both compounds with phosphatidylinositol, as calculated by conformational analysis, are similar [18]. Third, the inhibitory potency of gentamicin increases when tested on liposomes containing phosphatidic acid, phosphatidylserine or phosphatidylinositol, although equilibrium dialysis studies fail to reveal significant differences in the binding parameters of the drug towards these three types of negatively charged liposome [14]. Fourth, early studies ([12], see also Ref. 19 for review) showed that the number of cationic groups carried by an aminoglycoside is not the only determinant in its inhibitory potential even though it plays an important role. Altogether, these observations suggest that the

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binding of the drug to negatively charged lipids and the charge neutralization of the lipid bilayer it causes are not the only parameters governing the drug-induced inhibition of phospholipases. In a previous conformational study, we suggested that the accessibility of the drug bound to the bilayer to the hydrophilic phase could be as critical as the tightness of the binding. Unfortunately, we could not evaluate accurately the importance of this parameter.

In the present work, we have used a newly developed approach in the conformational analysis of lipids, namely the molecular hydrophobicity potential (MHP*) described by Brasseur [20] to study the accessibility of the aminoglycoside molecule to the water phase, considering first the isolated drug molecule and then the drug bound to a phospholipid bilayer. The MHP concept has been introduced to allow the visualization of the hydrophobic and hydrophilic envelopes around a peptide or a drug, including their three-dimensional representation by molecular graphics. This parameter is obtained by the calculation of the hydrophobicity potential around the molecule investigated. It has been used previously with success to classify the lipid-associating helices according to their molecular properties [20], as well as the structure of lipid–apolipoprotein complexes [21]. The aim of the present study is to give a molecular representation of the isopotential lines around the aminoglycosides in interaction with phosphatidylinositol. An isopotential line in the context of this paper links all zones in the space surrounding the drug where the hydrophobic–hydrophilic energy remains at an identical value. This approach allows visualization of the accessibility of the water molecule to the drug–phospholipid complex. We have used a series of different aminoglycosides (Fig. 1), with specific substitutions (in N1) and with variations in the number of amino groups (see position 2′). These molecules vary in their inhibitory potency towards lysosomal phospholipase A_1 as measured *in vitro*, and in their nephrotoxic potential. For comparison, we have investigated two other types of molecule, also shown in Fig. 1, namely a simple monoaminated sugar, 2-D-glucosamine, which does not bind to negatively charged liposomes and has no effect on lysosomal phospholipase activity, and bis(β-diethylaminoethylether)hexestrol (DEH), a diaminated amphiphilic drug, which binds to phospholipids and strongly inhibits the activity of lysosomal phospholipases [22].

MATERIALS AND METHODS

Aminoglycosides studied. The choice of molecules studied (kanamycin A, amikacin, gentamicin B, isepamicin, gentamicin C_{1a}, kanamycin B) was based on the following considerations. First, they all display the general structure of most aminoglycosides used nowadays in clinical practice, i.e. two aminosugars flanking a 2-deoxystreptamine [3]. Second, they belong to the two main sub-groups of these clinically used aminoglycosides, which carry either 4 or 5 amino groups (the additional amino-group being always in position 2′). Third, amikacin and isepamicin are both derivatives, of kanamycin A and gentamicin B, respectively, where the 1-N amino group is displaced away from the 2-deoxystreptamine. This substitution makes these molecules less susceptible to inactivation by bacterial enzymes [2, 3] (the most frequent mechanism of resistance to aminoglycosides), but also decreases their inhibitory potency towards lysosomal phospholipase and nephrotoxicity [12, 19]. Gentamicin C_{1a} was chosen among the three major components of the gentamicin complex as it does not carry a methyl group in position C6′ or N6′, and is therefore closer to the other aminoglycosides studied in this paper. Kanamycin B was chosen for the knowledge of the relationship between structure and toxicity for many derivatives of this drug [18], and because related compounds such as tobramycin and dibekacin are important pharmaceuticals which show excellent activity against *Pseudomonas aeruginosa* [1].

Determination of theoretical partition coefficient. This parameter was determined using the Rekker's table showing the fragmental hydrophobic constant (f) associated to various simple chemical fragments. The lipophilic character of the whole drug was then calculated as: $\log P = \sum_{x=1}^{N} a_x f_x$, where a is the number of similar fragments present in the molecule investigated and N the number of different types of fragments present. A negative value of log P is associated with a hydrophilic molecule whereas a positive value corresponds to a more hydrophobic structure.

Evaluation of the hydrophobic–hydrophilic balance and the distance between hydrophobic and hydrophilic centers. The transfer energy E_{tri} represents the free energy of transfer from a hydrophobic to a hydrophilic phase for a chemical function or an atom i. It is dependent upon the type of chemical bond of this chemical function or of this atom. The transfer energy per atom was calculated from the transfer energies compiled by Tandford [23] for a series of chemical analogues. Assuming that the molecular transfer energy is the sum of the transfer energy of the atomic constituents, we could derive E_{tri} values for the different hybridization states representing the atoms in an organic molecule. The atomic transfer energies E_{tri} are those normalized by Brasseur [20].

Hydrophobic–hydrophilic balance. This balance was defined as:

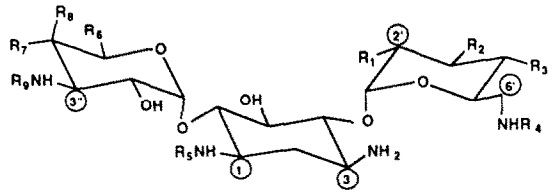
$$\Phi = \frac{\sum_{i=1}^{N} E_{tri}^{pho}}{\sum_{j=1}^{M} E_{trj}^{phi}} \qquad (1)$$

where E_{trj}^{phi} is the hydrophilic transfer energy of atom j and E_{tri}^{pho} the hydrophobic transfer of atom i.

Distance between the hydrophobic and hydrophilic centers. This distance (Δ) was defined as:

$$\Delta = | \overrightarrow{C_{phi}} - \overrightarrow{C_{pho}} |. \qquad (2)$$

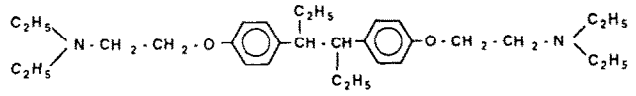
* Abbreviations: MHP, molecular hydrophobicity potential; DEH, bis(β-diethylaminoethylether)hexestrol.



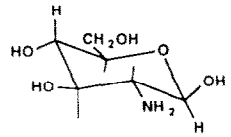
Aminoglycosides

	Substituants									log P.
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	
Kanamycin A	OH	OH	OH	H	H	CH_2OH	OH	H	H	- 6.673
Amikacin	OH	OH	OH	H	AHBA*	CH_2OH	OH	H	H	- 9.893
Gentamicin B	OH	OH	OH	H	H	H	CH_3	OH	CH_3	- 4.356
Isepamicin	OH	OH	OH	H	AHPA*	H	CH_3	OH	CH_3	- 8.103
Gentamicin C_{1a}	NH_2	H	H	H	H	H	CH_3	OH	CH_3	- 1.416
Kanamycin B	NH_2	OH	OH	H	H	CH_2OH	OH	H	H	- 6.613

* AHBA = L-aminohydroxybutyryl [-CO-CHOH-CH_2-CH_2-NH_2]
* AHPA = L-aminohydroxypropyl [-CO CHOH CH_2NH_2]



Bis (β-Diethylaminoethylether) Hexestrol



2-D-Glucosamine

Fig. 1. Structural formulae of the aminoglycosides and other compounds studied in this paper. For aminoglycosides, the values of log P (based on Rekker's Table) are indicated in the table.

The hydrophilic center $\overrightarrow{C_{phi}}$ was defined by:

$$\overrightarrow{C_{phi}} = \frac{\sum_{j=1}^{N} E_{trj}^{phi} \vec{r}_j}{\sum_{j=1}^{N} E_{trj}^{phi}} \quad (3)$$

in which $\vec{r}_j$ are the coordinates of the j atom and E_{trj}^{phi} the hydrophilic transfer energy of the atom j.

The hydrophobic center ($\overrightarrow{C_{pho}}$) was defined by the same equation, except that the hydrophobic transfer energies were taken into account. The calculation of the hydrophilic and hydrophobic centers requires the exact knowledge of the tridimensional structure of the molecule which, in the present case, was established by conformational analysis as explained briefly below.

Analysis of the conformation of the isolated molecule and of its orientation at the lipid–water interface. This analysis was performed as described extensively elsewhere [24–26]. The total conformational energy was calculated as the sum of the following terms: (i) the London–Van der Waals energy of interaction between all pairs of non-mutually bonded atoms, (ii) the generalized Keesom–Van der Waals interaction or electrostatic interaction between atomic point charges, (iii) the potential energy of rotation of torsional angles and (iv) the transfer energy of each part of the molecule.

Calculation of the MHP of the aminoglycoside molecule. This calculation was made based upon the "molecular lipophilicity potential" concept introduced by Furet *et al.* [27] and Fauchere *et al.* [28]. This parameter was defined by considering a molecule A surrounded by typical organic solvent molecules, and by assuming that the overall hydrophobicity is the sum of the hydrophobicity of its atoms. By analogy with the approach taken by Fauchere *et al.* [28], we assume that the hydrophobic interaction between a given point M in the space and an atom i exponentially decreases with the distance according to the equation:

$$\text{MHP} = E_{tri} \exp(r_i - d_i) \quad (4)$$

where E_{tri} is the transfer energy of the atom i, r_i is the radius of the atom i and d_i is the distance between the atom i and a point M [20]. In order to calculate the MHP, a cross-sectional computation was performed by 2 Å moves along the molecule. The MHP was then calculated with a precision of 2 Å using equation (4) for all points contained in this plane. A modification of the program developed for the visualization of the electrostatic isopotential lines [29] yielded a three-dimensional representation of the MHP around the drug molecule.

All calculations were performed on an Olivetti CP486 microcomputer equipped with an Intel 80486 processor, using the PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) procedures. Graphics were drawn with the PC-MGM+ (Molecular Graphics Manipulation) program.

RESULTS

The interaction of aminoglycosides with negatively charged phospholipids involves, besides the electrostatic and Van der Waals interactions, hydrophobic forces which also play an important role in the stabilization of the complexes formed. In a first step, the hydrophobic character of the aminoglycoside molecules investigated was estimated by the calculation of their theoretical partition coefficient (log P). The values obtained are indicated in Fig. 1. Gentamicin C_{1a} appeared as the most hydrophobic compound, followed by gentamicin B. Kanamycin B and kanamycin A were more hydrophilic. The introduction of a substituent in position N-1 of kanamycin A (4-amino-2-hydroxybutyryl) or of gentamicin B (3-amino-2-hydroxypropionyl), yielding amikacin or isepamicin, respectively, increased the hydrophilic character of the molecule.

In a second step, we have tried to derive information about the mode of insertion of the aminoglycoside molecules into a lipid matrix from the correlation observed between their hydrophobic–hydrophilic balance (Φ, Eqn 1) and the distance between their hydrophilic and hydrophobic centers (Δ, Eqn 2) [30] as illustrated in Fig. 2. All the aminoglycosides studied actually have a rather small value for Δ so that no meaningful correlation could be drawn between Δ and Φ for these drugs. In contrast, molecules capable of assembling themselves in organized structures such as phospholipids (e.g. dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylinositol) show a much greater value for Δ, together with a larger value for Φ, which places them far away from the aminoglycosides. Molecules such as DEH, or as an ionophore [lasalocide (X537A), the two structures (hydrophobic and interfacial) are shown on the Figure], that insert in bilayers, have an intermediate value of Δ. Determination of the MHP was therefore attempted to obtain a description of the hydrophobic envelope surrounding first the isolated molecules of aminoglycoside and then the drugs assembled with an increasing number of phosphatidylinositol molecules. Assembly was achieved by adding an

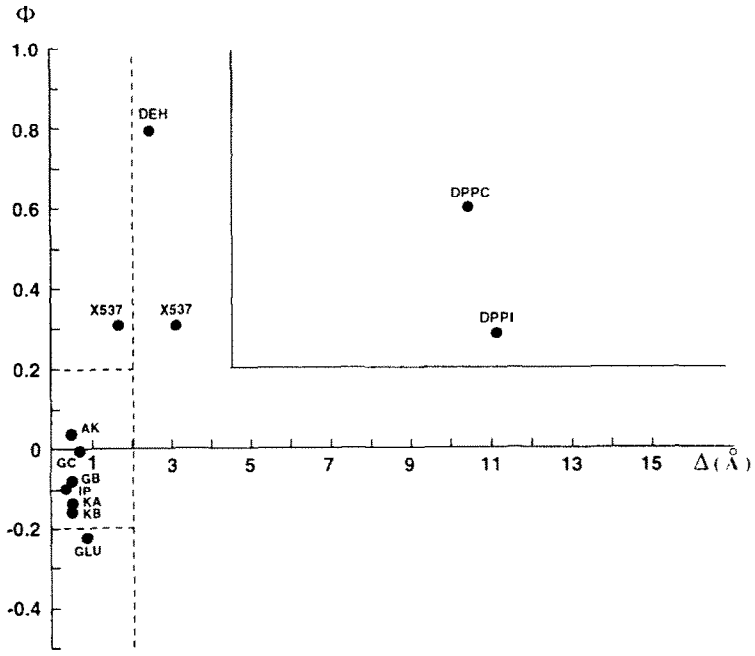


Fig. 2. Correlation between the hydrophobic–hydrophilic balance (Φ) as a function of the distance (Δ) between the hydrophobic and the hydrophilic centers of the aminoglycosides studied in this paper [amikacin, AK; gentamicin B, GB; kanamycin A, KA; kanamycin B, KB; isepamicin, IP; gentamicin C_{1a}, GC; glucosamine, GLU; lasalocide (an ionophore), X537; dipalmitoylphosphatidylcholine, DPPC; dipalmitoylphosphatidylinositol, DPPI]. For lasalocide, the positions of the two structures (hydrophobic and interfacial) are given.

increasing number of phosphatidylinositol molecules until the drug could no longer be accessed by an additional phospholipid. Drugs were analysed under their fully protonated form to mimic their behavior in lysosomes, the pH of which is estimated to lie at around 5.4 [31, 32], i.e. below the pK_a values of the amino groups of the aminoglycosides [33, 34]. Phosphatidylinositol was considered to carry one negative charge on its phospho group, assuming a pK_a of *ca.* 2.5 for this acid function. Figure 3 shows the MHP around the most probable conformer of phosphatidylinositol and of each aminoglycoside molecule (kanamycin A, amikacin, gentamicin B, isepamicin, gentamicin C_{1a} and kanamycin B), isolated and after the minimization procedure. For phosphatidylinositol, the picture reveals the amphipathic character of the molecule with the carbon 1 and 2 of glycerol (joining the two fatty acid chains) forming the boundary between the hydrophobic and hydrophilic domains. For aminoglycosides, examination of the hydrophobic envelopes revealed clear-cut differences between the various molecules investigated. Gentamicin C_{1a} appeared surrounded by a majority of hydrophobic envelopes whereas kanamycin B and kanamycin A were surrounded primarily by hydrophilic envelopes. In contrast, gentamicin B, isepamicin and amikacin were surrounded by both hydrophobic and hydrophilic envelopes, although amikacin appeared a more hydrophobic molecule than the other two aminoglycosides.

In the next step, we calculated the MHP of each aminoglycoside when surrounded by phosphatidylinositol. MHP isopotential lines around each aminoglycoside together with all first neighbouring phosphatidylinositol molecules are shown in panel A of Fig. 4. Panel B of the same Figure shows the MHP pictures for the drug itself, after having removed from the representation, but not from the calculations, the phosphatidylinositol molecules. Kanamycin A, kanamycin B and gentamicin B adopt, at the interface, a rather "flat" configuration with their N6′-N3″, N1-N3-N6′ and N6′-N3″ amino groups, respectively, almost in the same plane and oriented towards the hydrophobic plane, whereas the N3-N1, N2′-N3″ and N1-N3′ groups of the corresponding molecules face the aqueous phase. Among these aminoglycosides, gentamicin B appears more deeply inserted than the other two since the whole of the molecule is below the plane formed by the phosphorus atoms of phosphatidylinositol. The determination of MHP around kanamycin A shows that all the amino groups are close to both the hydrophobic and hydrophilic envelopes. For kanamycin B, the number of hydrophobic envelopes is low and the amino groups in position 6′ and 3″ appear entirely surrounded by hydrophilic envelopes. For gentamicin B, the amino groups located in position 1 and 3″ are surrounded by hydrophobic and hydrophilic envelopes whereas the amines in position 6′ and 3 are surrounded by hydrophilic envelopes only. In contrast, amikacin and isepamicin adopt a position parallel to the fatty acid chains with the 3-amino-3-deoxy-D-glucose moiety ("sugar moiety) much attracted towards the hydrophobic phase. The 6′ and 3 amino groups of amikacin and isepamicin points towards the aqueous phase, and the five nitrogen atoms of these molecules are surrounded by hydrophobic and hydrophilic envelopes. The hydrophobic envelopes around amikacin appear as more important than those around isepamicin. Gentamicin C_{1a} adopts the

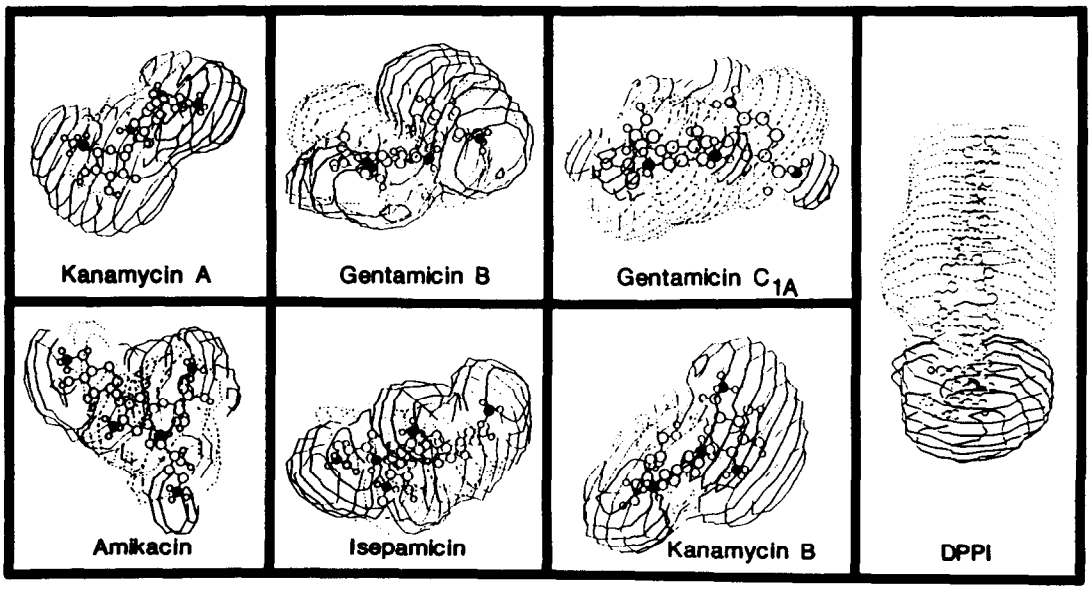


Fig. 3. MHP lines of isolated aminoglycosides studied in this paper and of phosphatidylinositol (DPPI). Hydrophilic envelopes are drawn in continuous lines whereas hydrophobic envelopes are represented by broken lines. The molecules are presented as stick–ball models to facilitate their observation. The nitrogen atoms of aminoglycoside molecules and the phosphorus atom of phosphatidylinositol have been stained in black.

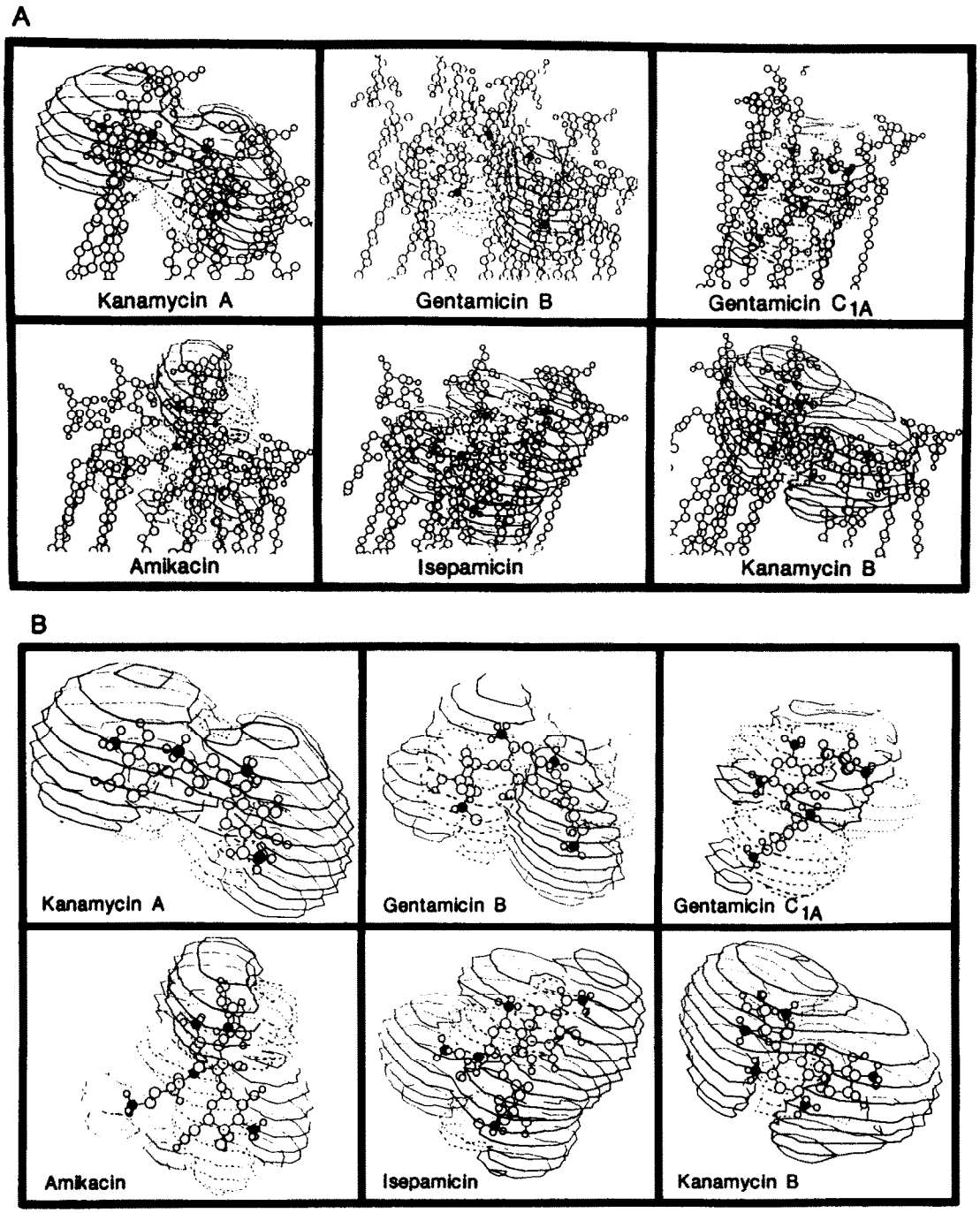


Fig. 4. MHP lines around the aminoglycosides assembled with phosphatidylinositol molecules. Hydrophilic envelopes are drawn in continuous lines whereas hydrophobic envelopes are represented by broken lines. The molecules are represented as the stick–ball models, with the nitrogen atoms in the aminoglycoside molecules stained in black. Panel A: representation of each drug molecule surrounded by the phospholipids; panel B: enlarged views of the aminoglycosides without representation of the phospholipids.

crescent shape described previously [15, 22] with its 2,6-diamino-2,6-dideoxy-D-glucose moiety ('sugar moiety) protruding into the lipid phase, and its 6' amino group inserted far into the bilayer. Gentamicin C_{1a} is almost entirely surrounded by hydrophobic envelopes. The exception is the amino group located in position 1, which is entirely surrounded by hydrophobic envelopes; all the other amino groups

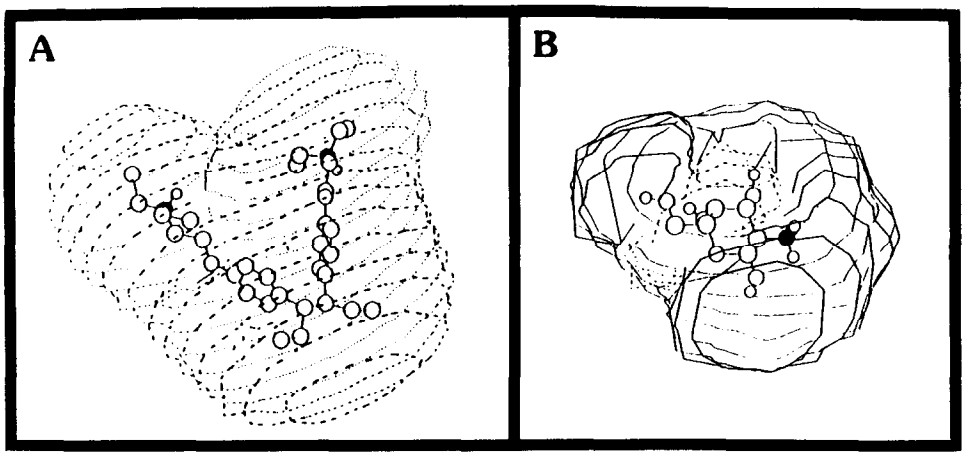


Fig. 5. Computer images of the MHP lines around isolated DEH (A) and glucosamine (B). Hydrophilic envelopes are drawn in continuous lines whereas hydrophobic envelopes are represented in broken lines. The nitrogen atoms are stained in black.

are actually surrounded by both hydrophilic and hydrophobic envelopes.

In contrast to the aminoglycosides, DEH was found to be totally surrounded by hydrophobic envelopes (Fig. 5) and therefore completely embedded in the phosphatidylinositol monolayer. Glucosamine was surrounded primarily by hydrophilic envelopes and no interaction with phosphatidylinositol could be evidenced.

DISCUSSION

Interactions between aminoglycosides and negatively charged phospholipids have been evidenced experimentally by Ca^{2+} displacement studies [16], gel filtration [6], equilibrium dialysis experiments [14, 35] and microelectrophoresis [36]. The complexes formed have been modelized by conformational analysis ([15, 17, 22, 37], see Ref. 19 for review). Although we so far have no experimental evidence that conformations of the aminoglycoside calculated in this way correspond to the physical reality, the techniques used were shown to predict correctly the actual conformations of Adriamycin® [38] or of the N-terminus of the paramyxovirus F_1 peptide [39] in interaction with phosphatidylinositol. Interactions between aminoglycosides and phospholipids, taking place *in vivo* in the lysosomes of kidney proximal tubular cells, are likely to be the initial event leading to inhibition of the activity of lysosomal phospholipases, renal phospholipidosis and, eventually, the toxicity of aminoglycosides towards the kidney (see Refs 9, 10 for reviews). Electrostatic binding (i.e. polar interactions between the phospho group of the phospholipids and the amino groups of the aminoglycosides) appear of primary importance in this respect [22, 40]. Yet, other factors, such as the Van der Waals interaction and the hydrophobic forces, could also be important. We suggested recently that the accessibility of the drug to the water phase and its ability to move easily between the bilayer and this phase could actually be most critical, although no specific approach to evaluate this parameter has been available up to now. Accessibility to water is not related simply to the overall hydrophobic character of an aminoglycoside. Indeed, experimental studies have shown that the introduction of increasingly bulky hydrophobic substituents into a specific position of an aminoglycoside, namely C-6″ of kanamycin B, does not significantly modify the inhibitory potency of this antibiotic [18, 41, 42]. However, we showed conversely that modulation of the hydrophobic–hydrophilic behavior of streptomycin (see derivatives described in Ref. 17), kanamycin A or gentamicin B (by the introduction of a substituent in N1) affects the inhibitory potential of the drugs [19]. These discrepancies probably result from the fact that determination of the theoretical hydrophobic–hydrophilic balance provides only a global representation of the hydrophobicity of a molecule, and is inadequate for the analysis of the complex interactions occurring between drugs and lipids. A more refined approach, namely the mapping of the aminoglycosides as a function of their hydrophobic–hydrophilic balance and of the distance between their hydrophobic and hydrophilic centers, failed to provide convincing clues, although this method distinguishes between molecules staying at the lipid–water interface and those becoming dissolved into the water or lipid phase [30].

The results presented in this paper show that application of the MHP concept, introduced previously for smaller molecules [27, 28] and for helical peptides [20, 21], may provide more pertinent albeit apparently surprising information than the methods discussed above. Thus, it is those molecules surrounded primarily by *either* hydrophobic (e.g. gentamicin C_{1a}) or hydrophilic (e.g. kanamycin B) envelopes which are strong inhibitors, whereas moderate inhibitors (e.g. amikacin, isepamicin) are surrounded by both types of envelope. The lack

of correlation with the hydrophobic–hydrophilic balance probably results from the fact that the MHP concept takes into account the three-dimensional structure of the molecule and, therefore, the effective interactions of each part of the molecule with the solvents. Thus, visualization by the MHP determination allows the acquiring of *molecular information* that is not acquired by the mere calculation of the hydrophobic–hydrophilic potential. It is difficult at this stage to interpret this finding in terms of rates and extent of drug–phospholipid association/dissociation, and more experimental studies need to be performed in this context. Yet, together with our previous findings concerning the aminoglycoside–phospholipid interaction (reviewed in Ref. 19), we may suggest that the inhibitory potential of an aminoglycoside towards lysosomal phospholipases, when interacting with negatively charged phospholipids included in a bilayer, is dependent upon: (i) the amount of drug bound [6]; (ii) the tightness and energy of interaction of this binding, which is primarily, but not exclusively, governed by the number of amino groups and by their position within the molecule [12, 15, 17]; (iii) the mode of insertion and orientation of the drug in the bilayer [15, 17, 37]; and (iv) the homopathic character (hydrophobic or hydrophilic) of the envelopes around the drug molecule. It will be of great interest in the future to determine the relative importance of these four parameters with respect to the inhibition of lysosomal phospholipases. This may, indeed, provide a more rational basis for the successful design of markedly less toxic aminoglycosides, a goal of great medical and biological interest which has not, so far, been met in spite of constant efforts [2, 43].

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