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Invited Review

**MOLECULAR PARAMETERS INVOLVED
IN AMINOGLYCOSIDE NEPHROTOXICITY**

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Aminoglycoside antibiotics are hydrophilic molecules consisting of an aminated cyclitol associated with amino sugar. They bind in vivo as well in vitro to negatively charged membranes. Their use as chemotherapeutic agents is unfortunately accompanied by ototoxic and nephrotoxic reactions, and the purpose of this review is to examine the role of the molecular interactions between aminoglycosides and membranes in the development of nephrotoxicity. ³¹P Nuclear magnetic resonance (NMR) and fluorescence depolarization have been used to characterize the effect of aminoglycosides on phosphate heads and fatty acyl chains of phospholipids. ¹⁵N NMR has been used to obtain interesting information on

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regioselective interactions of amino groups of antibiotics with phospholipids. The binding of aminoglycosides with negatively charged membranes is associated with impairment of phospholipid catabolism, change in membrane permeability, and membrane aggregation. Biochemical analysis and ^1H NMR spectroscopy have brought information on the molecular mechanism involved in the impairment of phospholipid catabolism. Nephrotoxic aminoglycosides could induce sequestration of phosphatidylinositol and therefore reduce the amount of negative charge available for optimal lysosomal phospholipase activity toward phosphatidylcholine included in liposomes that also contain cholesterol and sphingomyelin. Conformational analysis shows that aminoglycosides, which have a high potency to inhibit lysosomal phospholipase activity, adopt an orientation parallel to the lipid/water interface. This orientation of the aminoglycoside molecule at the interface is also critical to explain the marked increase of membrane permeability induced by less nephrotoxic aminoglycosides such as isepanamicin and amikacin. This effect is indeed only observed with aminoglycosides oriented perpendicular to this interface, probably related to the creation of a local condition of disorder. The impairment of phospholipid catabolism, which is considered to be an early and significant step in the development of aminoglycoside toxicity, is therefore not related to the change in membrane permeability. However, the role of this latter phenomenon and of membrane aggregation for aminoglycoside nephrotoxicity could be further investigated.

DRUG-MEMBRANE INTERACTION

The unraveling of the molecular nature of the interactions of drugs with membranes is both an old and a recent development in our knowledge of their activity or potential toxic effects. Thus, although a large number of studies have dealt in the past with the morphological study of cell membrane alterations induced by drugs, the increasing knowledge of membrane structures and functions gained over recent years has allowed examination of these interactions at a much deeper level than was feasible before.

The purpose of this review is to examine and review the interactions of a class of antibiotics, the aminoglycosides, with membranes in the hope of better understanding the mechanism of their toxicity. Aminoglycosides are toxic for proximal tubular cells of the kidney and the hair cells of the cochlea. In both cases, interaction of these drugs with membrane constituents has been suggested to play a major role in the onset of these toxic phenomena (Tulkens, 1986, 1989; Tulkens et al., 1990).

Biological membranes serve both to protect the cell from the outside environment and to perform essential functions in the cell by allowing for the selective transport of nutrient and other molecules. These functions are believed to be intimately related to the biophysical organization of the membrane, as reviewed in Cullis et al. (1983) and Yeagle (1989), which in turn strongly depends on the interactions between their main constituents, namely, lipids and proteins.

This organization can, however, be perturbed by the presence of drugs. Although the interactions of drugs with proteins have been studied extensively, much less is known about the interactions between drugs and lipids. Different aspects of the drug-lipid interactions, which may be investigated in terms of perturbation of the structure and properties of phospholipid bilayers, are summarized in Figure 1.

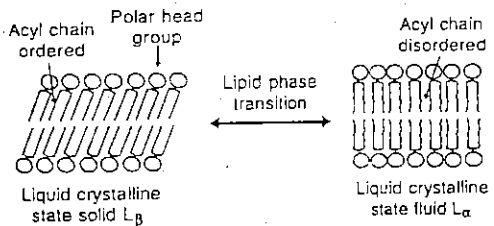
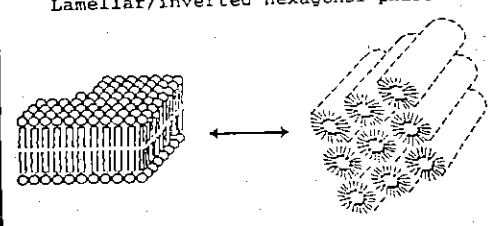
Parameters	Compound affecting	References
<p>Phase Transition Lamellar gel/liquid-crystalline phase</p>  <p>Acyl chain ordered Polar head group Acyl chain disordered Liquid crystalline state solid L_{β} Liquid crystalline state fluid L_{α}</p>	<p>β-blockers Opioid peptide Amphiphilic drugs</p> <p>Chlorpromazine Poly(-L-Lysine) Aminoglycosides</p> <p>Local anaesthetic dibucaine</p>	<p>Herbette et al, 1983 Ramaswani et al, 1992 Cater et al 1974 Kursch et al 1983 Hanpft and Mohr 1985 Thomas and Verkleij 1990 Borchardt et al 1991 Girke et al 1989 Luxnat and Galla 1986 Laroche et al 1988 Wang et al 1984 Ramsamy and Kaloyanides 1987 Van Bambeke et al 1993 Papahadjopoulos et al 1975</p>
<p>Phase Transition Lamellar/inverted hexagonal phase</p>  <p>Bilayer Hexagonal (H_{II})</p>	<p>Cardiotoxins from snake venoms Chlorpromazine Viral chemotherapeutic agents Adriamycin</p> <p>Mellitine Ellipticines Gramicidin</p> <p>Aminoglycosides Ca^{2+}</p> <p>Glycophorin Myelin basic protein</p>	<p>Batenburg et al 1985 Verkleij et al 1982 Epand et al 1987 Goormaghtigh et al 1982 Nicolay et al 1988 Batenburg et al, 1987 Nicolay et al 1988 Van Echteld et al 1982 Tournois et al 1990 Mingeot-Leclerq et al 1989 Vasilenko et al 1982 Verkleij et al 1982 Taraschi et al, 1982 Smith and Cornell, 1985</p>

FIGURE 1. Effects of various compounds on the physico-chemical properties of membranes.

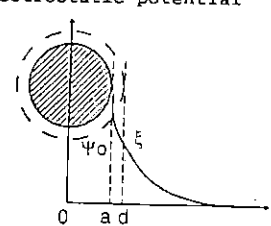
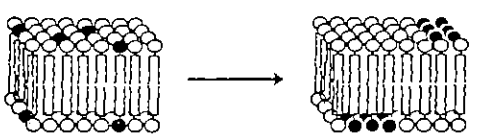
<p>Electrostatic potential</p> 	<p>n-alkanols anaesthetics Trypanozidal drugs (amphiphilic diphenyl methane derivatives) Aminoglycosides</p>	<p>Haydon and Elliott 1986 Lakhdar-Ghazal et al 1991 Chung et al 1985</p>
<p>Lateral phase separation</p> 	<p>Ca²⁺</p> <p>Poly-(L-lysine)</p> <p>Aminoglycosides Anthracyclines Vesicular stomatitis virus matrix protein</p>	<p>Silvius 1990 Van Dijck et al 1975 Kouaouci et al 1985 Galla & Sackmann 1975 Hui et al 1983 Hartmann et al 1977 Carrier and Pezolet 1986 Laroche et al 1988 Galla & Sackmann 1975 Hartmann & Galla 1977 Schanck et al 1992b Nicolay et al 1988 Wiener et al 1985</p>

FIGURE 1. (Continued) Effects of various compounds on the physico-chemical properties of membranes.

All these interactions can perturb the organization of the membrane and therefore alter its function. In parallel, these interactions can also correlate to either the biological effects of the drugs, for instance, the sodium channel (Lee, 1976) and anaesthetic compounds (Haydon & Elliott, 1986), or to their side effects, such as drug-induced lipidosis (Lüllmann et al., 1975; Hein et al., 1990), especially aminoglycoside antibiotics (Tulkens et al., 1990). The aim of this chapter is to try to understand the potential relationship between the interactions of aminoglycoside molecules with membranes and the nephrotoxicity induced by these antibiotics.

Aminoglycoside Antibiotics as an Example of Drug Interacting with Membranes

Aminoglycosides are potent, wide-spectrum antimicrobials. An excellent review of their chemical structures and main antibacterial properties has been published by Nagabhushan et al. (1982). Briefly, the family of aminoglycoside antibiotics is defined by the presence of two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring. The aminocyclitol ring is either a streptidine, as in streptomycin or dihydroxystreptomycin, or a 2-deoxystreptamine, as in kanamycin, gentamicin, and their derivatives.

The success of aminoglycosides as chemotherapeutic agents stems from (1) their spectrum, which covers most gram-negative bacteria and staphylococci, (2) their fast and dose-related bactericidal effect, and (3) the marked postantibiotic activity that they exert toward those bacteria that are not killed during first contact (Craig et al., 1988).

These useful properties, however, are shadowed by a narrow therapeutic index because of a significant occurrence of oto- and nephrotoxic reactions (Kahlmeier & Dahlager, 1984; Sande & Mandel, 1990; Lietman, 1985). The latter side effect can be explained by the fact that aminoglycosides are taken up by the proximal tubular cells of renal cortex and accumulate in their lysosomes, where they reach concentrations in the millimolar range (De Broe et al., 1984; Ciurtea-Marrion & Tulkens, 1986). This accumulation leads to phospholipidosis, by inhibition of the activities of lysosomal phospholipases (Laurent et al., 1982; Carlier et al., 1983; Mingnot-Leclercq et al., 1988, 1990a, 1991a), which may trigger cell necrosis (Laurent et al., 1990). Focal tubular necrosis, if not appropriately compensated by a commensurate regenerative process, will then cause acute tubular necrosis and renal failure (Tulkens, 1986, 1989; Laurent & Tulkens, 1987; Laurent et al., 1990).

Because of their polyaminated character, aminoglycoside molecules are expected to be fully protonated at lysosomal pH (i.e., around 5–6; Rejlingoud & Tager, 1977; Ohkuma & Poole, 1978). Since most cellular membranes (McGilveray, 1979), including those of lysosomes (Esko & Raetz, 1983), contain between 10 and 30% negatively charged phospholipids such as phosphatidylinositol, phosphatidylserine, phosphatidic acid, or phosphatidylglycerol, aminoglycosides could therefore bind to the membranes of

lysosomes and/or to the cellular membranes carried into lysosomes by autophagy (de Duve & Wattiaux, 1966). This binding is probably responsible for the inhibition of lysosomal phospholipase activity, which is the earliest event in the development of aminoglycoside nephrotoxicity.

The main goals of the performed studies are summarized in Figure 2 and consist of (1) the qualitative description of the interaction between aminoglycoside molecules and negatively charged lipids, (2) the effect of this interaction on biological phenomena such as enzymatic activity of lysosomal phospholipase, but also on fusion and permeability processes, and (3) the relation between alterations of these biological phenomena and cell necrosis induced by aminoglycosides.

In this study, we used artificial membranes to characterize and quantify the aminoglycoside-membrane interaction. We have generally taken a somewhat simplified model of membrane containing only phosphatidylinositol, phosphatidylserine, or phosphatidic acid, as a negatively charged phospholipid, and phosphatidylcholine, sphingomyelin, and cholesterol as "structural" lipids.

For this purpose, we compared the effect on phospholipid properties of various aminoglycosides (amikacin, kanamycin A and B, isepamicin, gentamicin B, gentamicin complex) that exhibit large differences in their nephrotoxic potential, as evaluated *in vitro* by their potential to inhibit lysosomal phospholipase A₁ and A₂ activity toward phosphatidylcholine (Laurent et al., 1982; Hostetter & Hall, 1982; Carlier et al., 1983; Mingeot-Leclercq et al., 1988, 1990a, 1991a, 1991b; Van Schepdael et al., 1991a, 1991b) and *in vivo* by their potential to induce phospholipidosis, phospholipiduria, and cell necrosis (Kosek et al., 1974; Aubert-Tulkens et al., 1979; De Broe et al., 1984).

INTERACTIONS BETWEEN AMINOGLYCOSIDES AND MEMBRANES

Binding of Aminoglycosides to Negatively Charged Phospholipids

The binding of gentamicin to lipid vesicles has been assessed by various techniques such as gel filtration (Laurent et al., 1982), equilibrium dialysis (Kishore et al., 1990a; Mingeot-Leclercq et al., 1990a), turbidimetry (Aramaki & Tsuchiya, 1989), ultracentrifugation (Kubo et al., 1986), microelectrophoresis (Alexander et al., 1979; Chung et al., 1985), displacement of Ca²⁺ bound to membranes (Lillman & Vollmer, 1982), determination of film expansion (Lohdt et al., 1976; Wang et al., 1984), and nuclear magnetic resonance of ³¹P (Mingeot-Leclercq et al., 1989), ¹⁵N (Schanck et al., 1992a) or ²H (Schanck et al., 1992b).

Using equilibrium dialysis at pH 5.4, Mingeot-Leclercq et al. (1990a) have shown that the maximal binding capacity of gentamicin to liposomes containing cholesterol, phosphatidylcholine, sphingomyelin, and a negatively charged phospholipid (phosphatidylinositol, phosphatidylserine, or phospha-

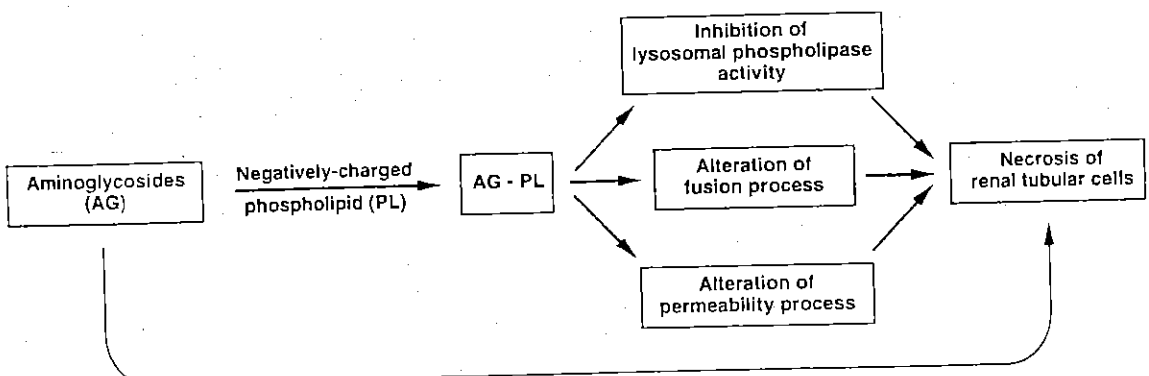


FIGURE 2. Phenomena involved in the development of aminoglycoside nephrotoxicity.

tidic acid) increases with the content of the membrane in the corresponding negatively charged lipid, up to a plateau value of ~ 0.3 mol of gentamicin per mol of acidic phospholipid. This result has been extended for vesicles containing only phosphatidylcholine and phosphatidylinositol (Pret et al., 1992). This shows that aminoglycosides may actually bind at a ratio of almost 1.5 positive charges per acidic phospholipid, which suggests that the drug may easily attract around itself several (three to four) of these lipids.

This interaction is mainly mediated by electrostatic interaction, since Laurent et al. (1982) and Chung et al. (1985) have shown that the binding is highly dependent on both the pH and ionic strength and requires the presence of a negatively charged lipid in the membrane. Thus, binding of aminoglycosides (pK_a values of amino groups between 6.0 and 9.5) to acidic phospholipids (pK_a values around 2.0–4.0) is optimal at pH 5.5 (Kishore et al., 1990a), and decreases at lower pH (where the phospho group becomes less negatively charged) as well as at higher pH (where the amino groups of the drug molecule lose their protons). The effect of pH explains why aminoglycosides may preferentially alter the properties of phospholipid bilayer in lysosomes compared to other subcellular structures, since lysosomes have a pH around 5.5 (Okhuma & Poole, 1978). Thus, most experimental studies were made at acidic pH. Moreover, by microelectrophoresis, Alexander et al. (1979) detected no influence of aminoglycosides on vesicle migration using neutral liposomes made from phosphatidylcholine only. This observation was confirmed for gentamicin using the techniques of turbidimetry, electrophoretic mobility determination, ultracentrifugation, and 2H NMR spectroscopy on vesicles containing phosphatidylethanolamine or phosphatidylcholine (Aramaki & Tsuchiya, 1989; Chung et al., 1985; Kubo et al., 1986; Schanck et al., 1992b).

The nature of the head group of the negatively charged phospholipid does not seem crucial, since no marked systematic difference between phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, or phosphatidic acid has been observed with respect to B_{max} or K_d values (the latter ranging between 3 and 47 μM) (Kubo et al., 1986; Mingeot-Leclercq et al., 1990a). However, the exceptions are noteworthy. Liposomes containing ganglioside GM₁ do not appreciably bind aminoglycosides, perhaps on account of steric hindrance (Chung et al., 1985), whereas liposomes containing phosphatidylinositol 4,5-diphosphate bind them to a greater extent (increased B_{max}) and with a higher affinity than liposomes containing phosphatidylinositol, phosphatidylserine, or phosphatidic acid, probably because of the larger density of negative charge around the polar head of this phospholipid (Aramaki & Tsuchiya, 1989).

Although the nature of the polar head of the acidic phospholipids seems relatively unimportant, the nature of the aminoglycosides markedly influences the binding of the molecule. Thus, the ability to cause charge reversal of liposomes prepared from phosphatidylinositol, or from a mixture of phosphatidylcholine and phosphatidic acid, and examined by microelectrophoresis, was

ranked in the following order: neomycin > gentamicin > tobramycin > amikacin > kanamycin A > streptomycin > dihydrostreptomycin, as shown by Alexander et al. (1979) and confirmed later by gel filtration data obtained by Carlier et al. (1983).

We next describe the interaction of an aminoglycoside with a negatively charged phospholipid in a molecular way. Since phospholipids have an amphiphilic character due to the presence of a polar or hydrophilic (water-loving) head group region as well as a nonpolar or hydrophobic (water-hating) region, it is interesting to study the interaction of the drugs with these two regions separately.

Experimental Insight into Interaction Between Aminoglycosides and Negatively Charged Phospholipids

Interactions of Aminoglycosides with Phosphate Groups of Phospholipids In most membranes, phospholipids are highly mobile in the plane of the membrane at 37°C (310 K). This mobility is an important property and has been extensively studied by ^{31}P NMR spectroscopy, in both model and biological membranes (Cullis & de Kruijff, 1976; de Kruijff et al., 1975). For example, narrow ^{31}P NMR spectra are obtained from sonicated phospholipid vesicles (de Kruijff et al., 1975; Berden et al., 1975), where the fast isotropic tumbling of the small vesicles is the dominant line-narrowing mechanism (McLaughlin et al., 1977). In contrast, unsonicated phospholipid liposomes give rise to much broader ^{31}P NMR spectra. In these systems, indeed, the isotropic averaging mechanisms are not effective due to tumbling and lateral diffusion of the phospholipid in the plane of the membrane, and the spectra obtained reflect the local anisotropic motions in the phosphate region of the polar headgroup (McLaughlin et al., 1977).

The influence of gentamicin on ^{31}P NMR spectra has been examined at both fixed and variable temperatures (Figure 3). At 298 K, addition of increasing amounts of gentamicin to unsonicated liposomes (composed of cholesterol, phosphatidylcholine, sphingomyelin, and phosphatidylinositol in a molar proportion of 5.5 : 4 : 5 : 2), decreases the narrow signal and steadily increases the broad asymmetric signal, with a low-field shoulder and a high-field peak, which indicates the presence of large bilayer structures (Figure 3A). Likewise, the interaction of aminoglycosides with the negatively charged phospho group of phospholipids can be measured by examining the decrease in effective chemical shift anisotropy ($\Delta\sigma$) (i.e., the chemical shift between the high-field peak and the low-field shoulder of the ^{31}P NMR broad spectra) upon raising the temperature. Due to increased motion upon warming, the effective chemical shift anisotropy steadily decreases when unsonicated control liposomes are warmed from 298 to 348 K. Compared to control liposomes, this decrease is significantly lower in the presence of gentamicin (Figure 3B). This result indicates that gentamicin is able to cause a significant restriction in the movements of the phosphate head of phospholipids. The method does not allow one to ascertain whether all or only a

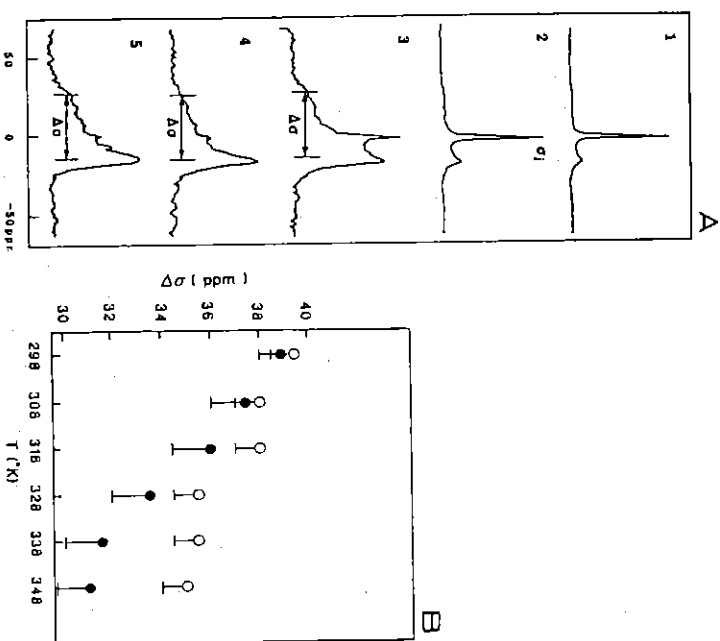


FIGURE 3. Interactions of aminoglycosides with phosphate groups of phospholipids. (A) ³¹P NMR spectra of unsaturated, negatively charged liposomes (cholesterol/phosphatidylcholine/sphingomyelin/phosphatidylinositol; molar proportion 5.5 : 4 : 5 : 2) (10 mg/ml) prepared in 4 mM acetate buffer, pH 5.4, and incubated 1 h at 37°C with increasing concentrations of genamycin (0, 0.11, 0.22, 0.32, and 0.43 mM). At 0 ppm σ is the chemical shift of phosphorus atoms undergoing isotropic motion and $\Delta\sigma$ the chemical shift between the high-field peak and the low-field shoulder of the ³¹P NMR broad spectra. To allow comparison among the different spectra, the intensity of the maximal signal is set on an identical value. (B) Variation of the effective chemical shift anisotropy $\Delta\sigma$ of liposomes control (●) or incubated 1 h at 37°C with 0.32 mM of genamycin (○) as a function of the temperature.

given proportion of phospholipids is affected in the mobility of their polar head. Because we saw that liposomes without negative charges do not bind aminoglycosides, it is likely that the decrease of mobility seen in negatively charged bilayers more preferentially affects the acidic phospholipids. Mobility could indeed be impaired simply by bridging acidic phospholipids together if complexes of an order greater than 1 : 1 (drug : phosphatidylinositol) are formed, as shown by the binding studies and also evidenced by the conformational analyses described later.

Interactions of Aminoglycosides with Negatively Charged Phospholipids Because of their cationic nature, it is obvious that it is the amino group of the aminoglycosides that must interact with the

phospho group of the acidic phospholipids. The question, actually, is more about which groups in the drug molecule and to what extent.

Specific interactions between individual aminated groups and a phospho group can be studied by ¹⁵N NMR spectroscopy using the natural abundance of ¹⁵N. Aminoglycoside nitrogens give highly resolved ¹⁵N spectra for isolated molecules in aqueous solution because of the rapid tumbling of these molecules in a nonviscous fluid, satisfying the extreme narrowing condition. An interaction between a given amino group and an acidic phospholipid such as phosphatidylinositol will therefore influence the ¹⁵N relaxation and the correlation times. Thus, in the presence of phosphatidylinositol, the correlation times will be governed by the reorientational motion of the phospholipid vesicles, the internal motions, and the exchange rate between the free aminoglycoside and the complex. If the correlation times become long enough, the extreme narrowing condition does not hold and the NMR signal, because of an important broadening, may disappear in the noise. This will be the case if the binding constant between the aminoglycoside and phosphatidylinositol vesicles is high enough or, in other words, if the interaction energy is important. When applied to aminoglycosides with different toxic potential, this approach has yielded very interesting results (Figure 4). Thus, the mixing of isepamicin—an aminoglycoside with a low toxic potential—with phosphatidylinositol has little influence on the ¹⁵N NMR spectrum, and we therefore conclude that the interaction between this drug and phosphatidylinositol vesicles should be weak. Conversely, the ¹⁵N signals of kanamycin B—a more toxic aminoglycoside—cannot be detected in the presence of phosphatidylinositol, which suggests tight binding of the antibiotic to the vesicles and a drastic reduction in its mobility. Amikacin and kanamycin A show an intermediate behavior, and are also known to be moderately toxic compared to kanamycin B. Several resonances are not perturbed in the presence of phosphatidylinositol, others are broadened or show heterogeneity, and some are not detectable. We interpret the broadening of the N-1 and N-3 resonances in the kanamycin A spectrum by assuming an intermediate exchange rate between the free antibiotic and the complex, whereas the N-6' amino group, remaining more mobile, still gives a narrow signal. Conversely, the very broad signal of N-3'', which is more tightly bound to phosphatidylinositol, disappears in the noise.

Interactions of Aminoglycosides with the Acyl Chains of Phospholipids Although there is no doubt that aminoglycosides strongly interact with the hydrophilic domain of the phospholipid bilayers, much less is established concerning a potential interaction with the hydrophobic domain. A direct interaction is unlikely in view of the polar character of the aminoglycosides, but the perturbations of the hydrophilic domain could lead to changes in the hydrophobic region. The most important property of the hydrophobic domain is its fluidity.

When applied to membranes, the term *fluidity* is usually used in a qualitative sense, and is generally meant to be a measure of the molecular resistance to movements in the membranes. Generally, fluidity is measured by

during this time, there will be no preferred orientation of the electric field vector of the emitted light, and the polarization will be zero. Polarization values between these two extremes could represent either a slow motion where the molecules do not have enough time (in 10^{-8} s) to randomize, or, alternatively, a rapid but constrained motion, which prevents randomization.

1,6-Diphenyl-1,3,5-hexatriene (DPH), a polyene hydrocarbon, is the most frequently used fluorescent probe because it partitions very favorably into the hydrocarbon region of lipids, has an intense fluorescence, does not appear to bind to proteins, and is sensitive to the membrane physical state. The membrane fluidity is examined at varying temperatures, and should cover the transition zone between the lamellar gel and liquid crystalline phases, since this zone is most profoundly affected by a change in membrane fluidity (Shinitzky & Barenholz, 1974).

When liposomes contain cholesterol (33%), no transition temperature can be evidenced and the polarization steadily and almost linearly rises approximately from 0.15 to 0.30 when the temperature is decreased from 55 to 12°C. In this system, gentamicin exerts no significant effect (Mingeot-Lecercq et al., 1989). In contrast, for liposomes without cholesterol and composed only of one type of phospholipid (preferably a synthetic one, such as dimyristoylphosphatidylcholine, which we have used), a very abrupt change in polarization with a transition temperature around 22°C is observed (Figure 5). Addition of phosphatidylinositol (PI) to dimyristoylphosphatidyl-

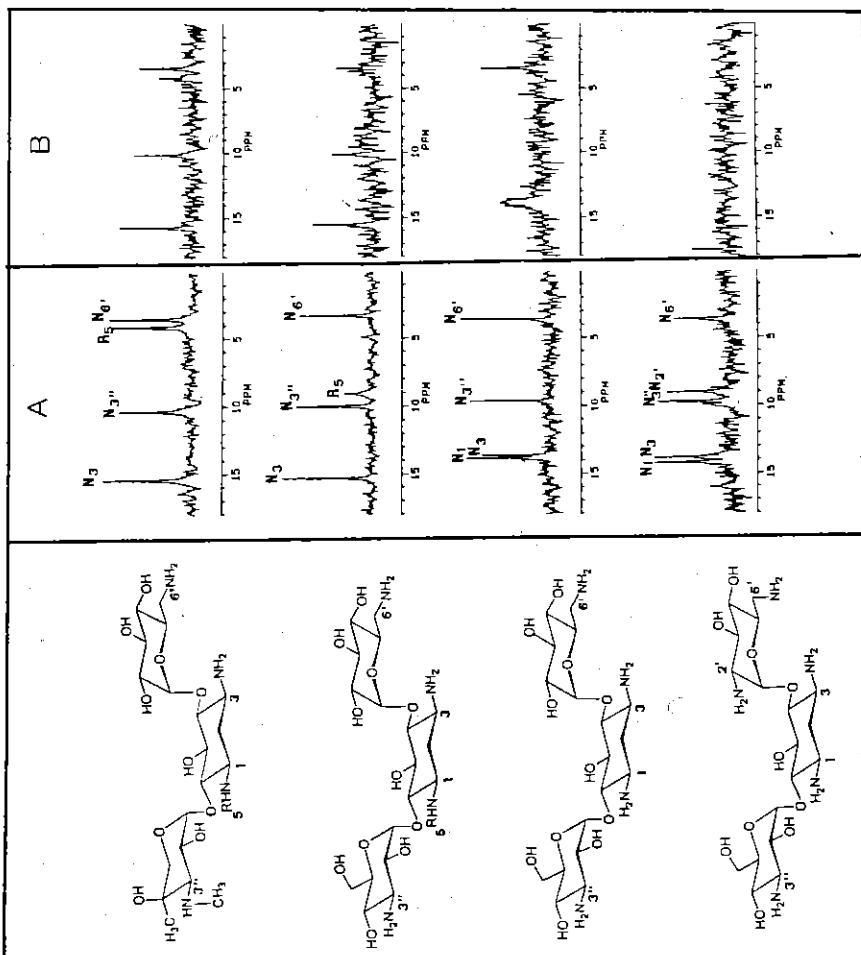


FIGURE 4. Interactions of amino groups of aminoglycosides with negatively charged phospholipids. Structural formulas of (A) isepamicin, amikacin, kanamycin A, and kanamycin B (from top to bottom) facing ^{15}N NMR spectra of these aminoglycosides in aqueous solution (5000–10,000 FIDs accumulated) or (B) mixed at 20 mol% in a phosphatidylinositol dispersion (40,000–60,000 FIDs accumulated).

observing the motion of a spin probe (electron spin resonance, ESR) or a fluorescent probe (fluorescence depolarization) incorporated in the bilayer. In the latter method, one excites a subpopulation of the probe molecules with polarized light. After a few nanoseconds, most of the excited molecules will emit a photon. If the molecules remain immobile during the time interval between the absorption and emission process (usually 10^{-8} s), the emitted light will be in a plane essentially similar to that of the light used for the original excitation. Conversely, if the molecules rotate isotropically and rapidly

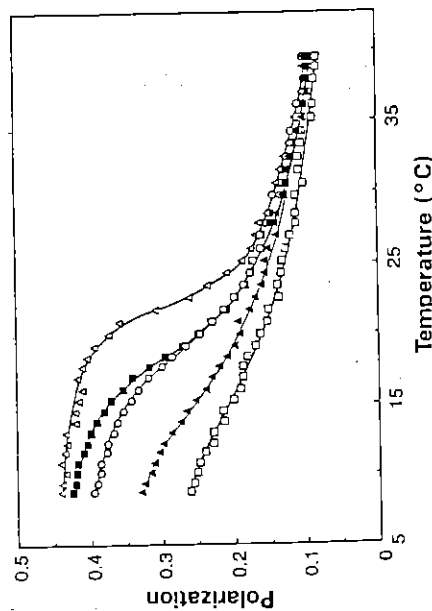


FIGURE 5. Interactions of aminoglycosides with acyl chain of phospholipids. Temperature dependence of the polarization of diphenylhexatriene included in liposomes made of dimyristoylphosphatidylcholine only (Δ); dimyristoylphosphatidylcholine + phosphatidylinositol [81.7% : 18.3% molar ratio] in the absence (\circ) or in the presence (\circ) of isepamicin; and dimyristoylphosphatidylcholine + phosphatidylinositol [63.4% : 36.6% molar ratio] in the absence (\square) or in the presence (\square) of isepamicin. The drug to average lipid molar ratio investigated was 100.

choline (DMPC) decreases the amplitude of the transition, which also occurs at lower temperatures (18.2 and 16.0°C for a PI content of 18.3% and 36.6%, respectively). Addition of an aminoglycoside (isepamicin) to liposomes made of only DMPC has no effect on the shape of the polarization curve but modifies that observed with DMPC/PI liposomes. The drug caused not only a less marked decrease of the amplitude of the transition, but also an increase in the apparent transition temperature (19.6 and 18.3°C for a PI content of 18.3% and 36.6%, respectively). Thus, isepamicin causes the thermotropic behavior of DMPC/PI liposomes to become closer to that of pure DMPC liposomes. Yet no difference of behavior between aminoglycosides can be evidenced by this approach.

Theoretical Insight into Interaction Between Aminoglycosides and Negatively Charged Phospholipids

Conformational Analysis and Hydrophobic Molecular Potential Understanding the functions of biological membranes and the perturbations by drugs at the molecular level requires a detailed knowledge of the possible and preferred conformations of the phospholipid molecules and of the mode of insertion of these drugs into the lipid matrix. This can be approached by the method of conformational analysis, a technique now made possible by the development of appropriate software [PC-TAMMO+ (theoretical analysis of molecular membrane organization; Brasseur, 1990) and PC-MSA+ (molecular structure analysis)]. This technique has proven useful in the description of the interaction of several drugs and sterols [aminoglycosides (Brasseur et al., 1984, 1985a; Tulkens et al., 1990), phorbol esters and diacylglycerols (Deleers et al., 1990), adriamycin and ethidium bromide (Goormaghtigh et al., 1990), antimycotics (Brasseur et al., 1983; Marichal et al., 1990), propranolol (Brasseur et al., 1985c; Chatelain & Brasseur, 1990) and alcohols (Brasseur et al., 1985b)], as well as of ionophores, carriers (Brasseur et al., 1982; Deleers & Brasseur, 1990) channel formers such as gramicidin A (Brasseur et al., 1987), and proteins (Brasseur, 1988; Brasseur et al., 1992) and peptides (Brasseur et al., 1986, 1988, 1990) with membranes. The method used takes into account the changes in the dielectric constant and the free energy transfer (also called solvation energy) when the molecule moves from one environment (hydrophobic phase) to another (hydrophilic phase) at the simulated lipid-water interface. The approach involves a two-step procedure consisting of the calculation of the stable conformation and orientation of the isolated molecules of phospholipid and drugs at the lipid-water interface and the calculation of the conformation of the drug molecules inserted in a lipid monolayer (Brasseur, 1990).

The results of the conformational analysis of the isolated aminoglycoside molecule at a lipid-water interface and stereo views of the most probable conformers, as well as their mode of assembling into a monolayer of phosphatidylinositol, phosphatidylserine, and phosphatidic acid, have been published by us previously (Mingeot-Leclercq et al., 1990b). A maximum of four or five

molecules of these phospholipids could be assembled around each aminoglycoside molecule (depending on each derivative studied). Figure 6 (left panel) illustrates the most probable location and orientation of phosphatidylinositol molecules assembled around kanamycin B, gentamicin C_{1a}, amikacin, and isepamicin and shows how each of these drugs interacts very differently with phosphatidylinositol molecules. In accordance with the experimental results described earlier, it is seen that drugs can be entirely maintained in the hydrophilic zone of the monolayer. In addition, several parameters appear important for the description of the interaction between aminoglycosides and

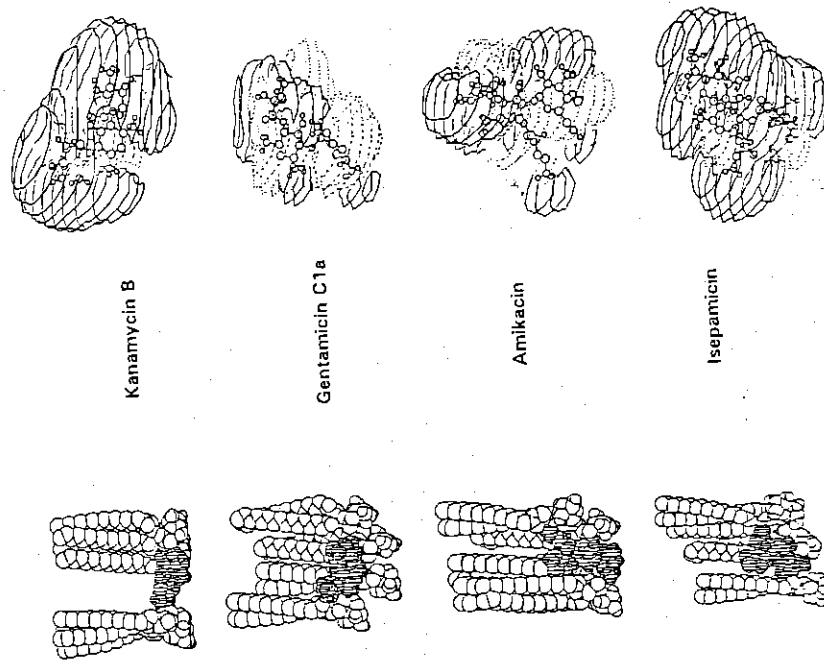


FIGURE 6. Theoretical insight of interaction between aminoglycosides and negatively charged phospholipids. Left: Comparative conformations of the mixed monolayers of phosphatidylinositol and kanamycin B, gentamicin C_{1a}, amikacin, and isepamicin under their full atomic sizes. Right: Molecular hydrophobic potential lines around aminoglycosides assembled with phosphatidylinositol molecules (not represented). Hydrophilic envelopes are drawn in continuous lines, and hydrophobic envelopes are represented by broken lines.

phospholipids. Among them, the depth of the insertion, the orientation of the drug relative to the lipid-water interface, and the calculated energy of interaction between the drug and the phospholipid seem crucial.

Taking as an example the four aminoglycosides shown in Figure 6 (left panel), it is clear that kanamycin B and gentamicin C_{1a}, which both are toxic, tend to adopt an orientation parallel to the interface (and also have a high interaction energy, of the order of -47 kJ/mol), whereas amikacin and isepamicin, which are less toxic, adopt a perpendicular orientation and also have a lower energy of interaction (-35 kJ/mol). The respective roles of these parameters with respect to toxicity have been elaborated further in Brasseur et al. (1984, 1985a) and Tulkens et al. (1990).

Thanks to a newly developed approach in the conformational analysis of lipids, namely, the molecular hydrophobicity potential (MHP) described by Brasseur (1991), we are now able to study the accessibility of a drug inserted into a lipid bilayer to water phase. 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 with success to classify the lipid-associating helices in peptides according to their molecular properties (Brasseur, 1991), as well as the structure of lipid-apolipoprotein complexes (Brasseur et al., 1992) and the stability of insertion of the dexamethasone palmitate into the lipid layer (Benamer et al., 1993). The molecular representation of the isopotential lines (i.e., the lines that link all zones in the space surrounding the drug where the hydrophobic-hydrophilic energy remains at an identical value) around aminoglycosides in interaction with phosphatidylinositol (Figure 6, right panel) allows visualization of the accessibility of the water molecule to the drug-phospholipid complex. Our studies have indicated that aminoglycosides can be surrounded either by isopotential hydrophobic envelopes (e.g., gentamicin C_{1a}) or by isopotential hydrophilic envelopes (e.g., kanamycin B), but also by both types of envelopes (e.g., amikacin and isepamicin). 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 are needed in this context.

Yet these results could be important to explain the variable potential of aminoglycosides to inhibit the lysosomal phospholipase activity or to alter the membrane permeability (discussed later).

EFFECTS OF THE INTERACTIONS BETWEEN AMINOGLYCOSIDES AND MEMBRANES ON BIOLOGICAL PHENOMENA

Effect of the Binding of Aminoglycosides to Negatively Charged Membranes on Lysosomal Phospholipase Activity

Aminoglycosides inhibit the activities of lysosomal phospholipases toward both neutral and acidic phospholipids. The latter effect is easily

understandable if one considers that aminoglycosides bind to these lipids. An effect on neutral phospholipids such as phosphatidylcholine is less easy to understand. Yet phosphatidylcholine and phosphatidylethanolamine are both major constituents of membranes and are found in large excess in aminoglycoside-treated cells or in the kidney of animals treated with these drugs.

The role of the binding of aminoglycosides to negatively charged membranes in the inhibition of lysosomal phospholipase activity toward phosphatidylcholine has been critically examined. Carlier et al. (1983) have shown a direct relation between the amount of gentamicin, amikacin, or streptomycin bound to phosphatidylinositol-containing liposomes and the inhibition of the activity of lysosomal phospholipases toward phosphatidylcholine. In addition, a reduction of the number of cationic groups in aminoglycosides, the substitution of the N-1-amino function of kanamycin A or of gentamicin B by an aminohydroxybutyl or aminohydroxypropionyl side chain, or the substitution of the position C-1 of gentamicin C₂ by a hydroxymethyl group decreases both the binding and the inhibitory potency of the drug toward lysosomal phospholipase activity (Carlier et al., 1983, 1986, 1988; Brasseur et al., 1984). Addition of polyaspartic acid—which binds the aminoglycosides and can therefore be used to prevent their association to acidic phospholipids—also reduces their inhibitory potential (Kishore et al., 1990a, 1990b, 1992). Finally, under conditions where no binding of aminoglycoside is detected, such as toward free zwitterionic phospholipids or neutral liposomes (Austlander et al., 1975; Laurent et al., 1982; Carlier et al., 1983; Ganesan et al., 1983; Kirschbaum, 1984; Chung et al., 1985; Ramsammy & Kaloyanides, 1985), no inhibitory effect on the activity of lysosomal phospholipases is observed (Mingeot-Leclercq et al., 1988; Hostetter & Jellison, 1990).

Actually, two *in vitro* models of inhibition of phospholipase A₁ activity toward phosphatidylcholine induced by aminoglycosides, the *charge neutralization model* (Mingeot-Leclercq et al., 1988, 1990a, 1991a; Piret et al., 1992) and the *substrate depletion model* (Hostetter & Jellison, 1990; Piret et al., 1992) have been proposed. In the first model, aminoglycosides bind to the negatively charged phospholipid and thereby reduce the amount of negative charges present in the vicinity of the substrate (phosphatidylcholine) and required for optimal activity. In the second model, aminoglycosides bind to the phosphatidylinositol and interfere with the access of the enzyme to the surface of the lipid vesicle. These two models result from the fact that the environment of phosphatidylcholine in lipid vesicles (presence of cholesterol and/or sphingomyelin) influences markedly the activity of phospholipase A₁, its regulation by the presence of negatively charged phospholipids, and the nature of its inhibition by aminoglycosides.

The role of lateral organization of lipids for inhibition of lysosomal phospholipases induced by aminoglycosides has been further examined by ²H NMR spectroscopy using liposomes containing a mixture of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (PI). Deuterium can be substituted for hydrogen at specific or at all positions in the lipid molecule, and

this relatively benign substitution is generally considered as nonperturbing compared to the technique using probes embedded in the bilayer. The quadrupolar splitting $\Delta\nu_Q$ is dependent on the time-averaged orientation between the C-D bond vector and the bilayer. This time-averaged orientation is usually quantified in terms of an order parameter.

A qualitative measure of the effect of aminoglycosides on acyl chains order may then be obtained from the spectral moments M_1 and M_2 , which are related to the average and mean squared order parameter, respectively, of the acyl chains. Both are studied at increasing temperatures, and their values show a similar and marked decrease at the transition temperature. Only the values obtained for M_2 are given in Figure 7. Differences in the spectral behavior become manifest with respect to the temperature dependence of the spectral moments when an equimolar mixture of perdeuterated dipalmitoylphosphatidylcholine and of phosphatidylinositol (Figure 7B) is incubated with kanamycin A or kanamycin B (nephrotoxic aminoglycosides with high inhibitory potency of lysosomal phospholipase), but not with isepamicin or amikacin (less nephrotoxic compounds with low inhibitory potency toward lysosomal phospholipase). These results may be interpreted as follows: In the presence of kanamycin A and B, the phosphatidylinositol binds the amino-glycoside molecule and forms aminoglycoside-phosphatidylinositol domains, leaving the lipid matrix effectively enriched in phosphatidylcholine. Below the DPPC transition temperature, the phosphatidylcholine-enriched domains form a gel phase detected by the broad component, whereas above the transition temperature the average acyl chains order parameter of each mixture is essentially the same. In contrast, addition of aminoglycosides to multilamellar dispersions of perdeuterated dipalmitoylphosphatidylcholine induces almost no effect on the variation of the first and second moments of ^2H NMR spectra compared to controls (Figure 7A). The results indicate therefore that the antibiotics have a minor influence on the motional and structural characteristics of phosphatidylcholine in a bilayer. Similar results were obtained for unsaturated 1-palmitoyl-2-oleoyl phosphatidylcholine (Schanck et al., 1992b). Unfortunately, no data are available for other aminoglycosides, such as gentamicin or streptomycin. However, the role of lateral organization of lipids for inhibition of lysosomal phospholipase induced by aminoglycosides has been questioned, and it is thus reasonable to propose that the sequestration of phosphatidylinositol is one event involved in the inhibition of lysosomal phospholipase activity.

Our understanding of the molecular mechanisms involved in the inhibition of lysosomal phospholipase activity by aminoglycosides can be improved by taking into account the results of the conformational analyses described earlier. In this context, the depth of the insertion, the orientation of the drug relative to the lipid-water interface, the energy of interaction, and the accessibility of the aminoglycoside molecule to the aqueous phase appear critical, in the sense that a low degree of insertion corresponding to a relatively weak energy of interaction, a position of the drug parallel to the fatty acid chains,

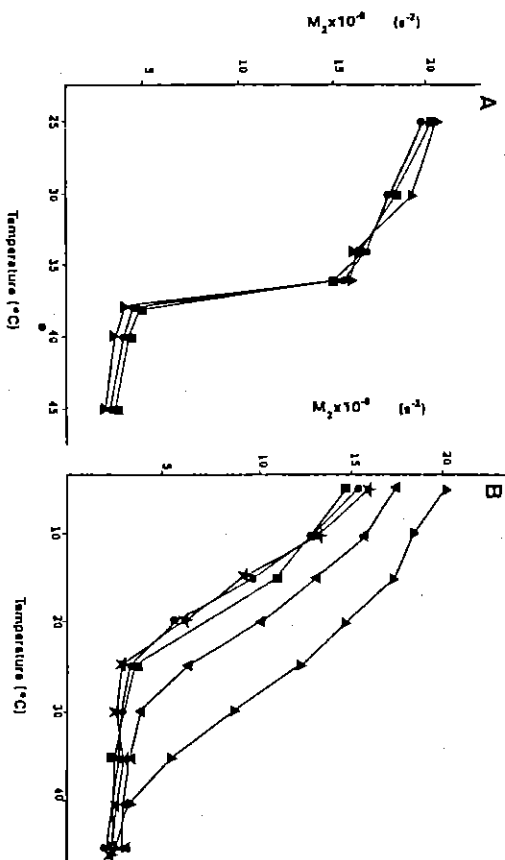


FIGURE 7. Effect of aminoglycosides on lateral phase separation of lipids. (A) Temperature dependence of the second moment M_2 of ^2H NMR spectra: ●, DPPC-d62; ▲, DPPC-d62 + 40% kanamycin A; □, DPPC-d62 + 40% isepamicin. (B) Temperature dependence of the second moment M_2 of ^2H NMR spectra of DPPC-d62/PI equimolar mixtures and after addition of 20 mol% aminoglycoside: ●, DPPC-d62/PI; ■, + isepamicin; ▲, + amikacin; ▲, + kanamycin A; ▼, + kanamycin B.

and the presence of both hydrophobic and hydrophilic envelopes around the aminoglycoside molecule are associated with a low inhibitory potential.

Effect of the Binding of Aminoglycosides to Negatively Charged Membranes on the Fusion Process

Effect of Aminoglycosides on Lipid Polymorphism It has been recognized that factors that tend to induce nonbilayer structures will also induce fusion between membrane systems (Ellens et al., 1989; Eppard, 1990).

Electron microscopy of freeze-etched lipids (Cullis et al., 1978) is classically used to observe and characterize nonbilayer structures, but this approach has not yet been developed with aminoglycosides. Phospholipids in the H_{II} hexagonal phase, however, give rise to a distinctly different ^{31}P NMR spectrum than do phospholipids that are in a lamellar phase (Cullis et al., 1978, 1983; Cullis & de Kruijff, 1978a, 1978b) (Figure 8, left panel). Bilayer systems exhibit broad, asymmetric ^{31}P NMR spectra with a low-field shoulder and high-field peak separated by about 40 ppm, whereas H_{II} phase systems exhibit spectra with reverse asymmetry that are narrower by a factor of two, due to the possibility of additional motional averaging in these structures arising from the rotation of the cylinders along their long axis. Using this technique, we have shown that liposomes made of dioleoyl phosphatidylethanolamine and diethyloleoylphosphatidylethanolamine (1 : 1, w/w)

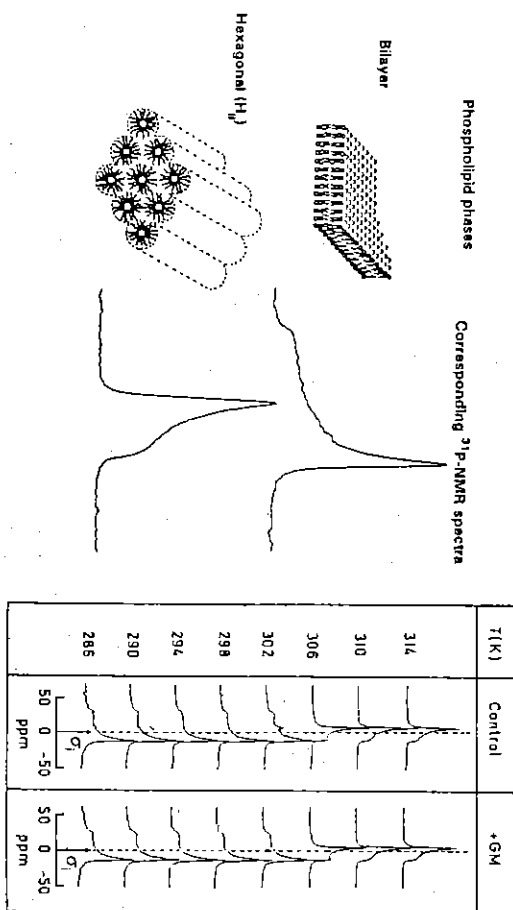


FIGURE 8. Effect of aminoglycosides on lipid polymorphism. Left: ^{31}P NMR spectra of phospholipids in various phases. Bilayer systems exhibit broad, asymmetric ^{31}P NMR spectra with a low-field shoulder and high-field peak separated by about 40 ppm, whereas H_{II} phase systems exhibit spectra with reversed asymmetry that are narrower by a factor of two. Right: ^{31}P NMR spectra of liposomes made of dioleoyl phosphatidylethanolamine and dioleoyl phosphatidylethanolamine (1 : 1) at increasing temperature. Gentamicin (GM) was added at a final ratio of drug to lipids of 1 : 3. The 0 ppm value is set at σ , corresponding to the isotropic shift value.

upon warming displayed a marked temperature transition between 302 and 306 K (Figure 8, right panel). 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 behaved as controls, 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 (Cullis et al., 1978). This negative result, however, should not be considered as definitive, since so far no study has used vesicles containing an acidic phospholipid, without which aminoglycosides probably do not bind to the membrane. Recently, we have shown that, upon warming, aminoglycosides: in interaction with negatively charged liposomes prevent the spontaneous formation of inverted micelles, intermediate structures observed during fusion process (Van Bambeke et al., submitted).

Effect of Aminoglycosides on the Size and Morphology of Liposomes

The apparent average diameter of the liposomes can easily be evaluated by quasi-elastic light spectroscopy (Mazer et al., 1979). In this procedure, the Brownian motion of the vesicles causes temporal fluctuations in the scattered

intensity. The average diffusion coefficient of the vesicles can then be obtained from a time-correlation function of the scattered light intensity, and it is possible to deduce the apparent population size. The determination of the real, individual diameter of the particles requires, however, the direct observation of replicas of freeze-etched samples in the electron microscope (Moor et al., 1961; Bachmann & Schmitt, 1971), an approach that is much more time-consuming.

Actually, addition of gentamicin results in a significant increase of the apparent size of phosphatidylinositol-containing liposomes, and this effect is abolished with the omission of acidic phospholipid (Mingeot-Leclercq et al., 1989). This increase of liposome apparent size in the presence of gentamicin is confirmed by analysis of the ^{31}P NMR spectra at a fixed temperature of 298 K, which shows a dual signal in which a broad component with a low-field shoulder and a high-field peak was superimposed on the isotropic signal (Mingeot-Leclercq et al., 1989). Electron microscopic studies (Figure 9), however, showed that all these effects mostly resulted from an aggregation of the vesicles rather than from a real increase in size, as shown by the morphological appearance of replicas prepared from freeze-etched liposomes (Mingeot-Leclercq et al., 1989). In contrast to what was seen with a fusio-



FIGURE 9. Morphological appearance of negatively charged, sonicated liposomes (cholesterol/phosphatidylcholine/sphingomyelin/phosphatidylinositol; molar proportion 5.5 : 4 : 5 : 2) (10 mg lipid/ml) prepared in 4 mM acetate buffer, after freeze-etching. (A) Control liposomes.

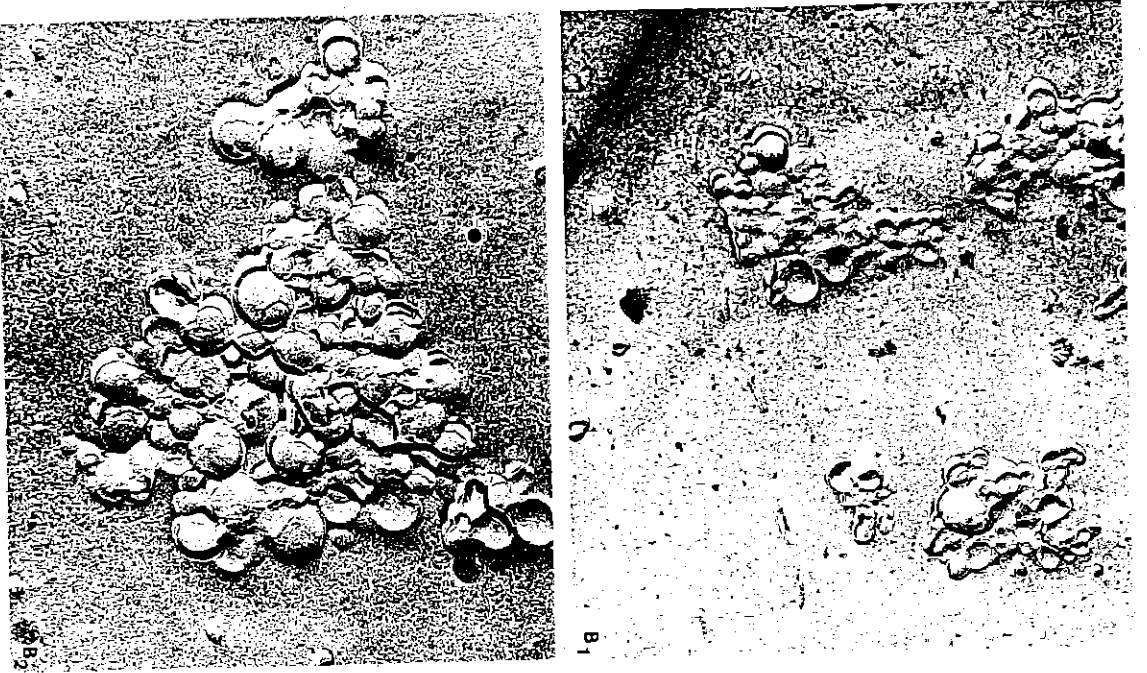


FIGURE 9. (Continued) Morphological appearance of negatively charged, sonicated liposomes (cholesterol/phosphatidylcholine/sphingomyelin/phosphatidylinositol; molar proportion 5.5 : 4 : 5 : 2) (10 mg lipids/ml) prepared in 4 mM acetate buffer, after freeze-etching. (A) Liposomes incubated with gentamicin (0.32 mM). Magnifications: A, B_1 , 37,500 \times ; B, 85,000 \times .

genic agent such as bis beta (diethylamino ethyl) hexestrol, no lipidic particles, representing regions of inverted micelles within the bilayer and that play a role in membrane fusion (Cullis et al., 1983; Verkley, 1984) were observed (Mingeot-Leclercq et al., 1989). The lack of fusogenic effect of gentamicin toward liposomes has been confirmed (Van Bambeke et al., submitted) by studies investigating the effect of the drug on the fusion of the lipidic phase, using the energy transfer assay (Struck et al., 1981) and the relief of fluorescence self-quenching of octadecyl rhodamine B chloride (Hoekstra et al., 1984) and by ^{31}P NMR.

Effect of Aminoglycosides on Lysosomes—Pinocytic Vesicle Fusion in Rat Kidney Proximal Tubules

The effect of gentamicin, at low doses, on the capacity of lysosomes to fuse with other intracellular vacuoles has been examined by using horseradish peroxidase as a marker of endocytic activity and of the process of lysosome-phagosome fusion during the handling of exogenous proteins. Girgea-Marion et al. (1986) showed that a treatment with gentamicin in kidney tubular cells did not significantly modify the intracellular accumulation of horseradish peroxidase, but largely modified its fate. Thus, in rats not exposed to gentamicin, more than half of the lysosomes contained horseradish peroxidase activity ~ 3 h after its administration. In animals treated with gentamicin, only one-third of the lysosomes exhibited horseradish peroxidase activity, and lysosomes overloaded with phospholipids—a sign that gentamicin had accumulated in them and had caused perturbation of phospholipid catabolism—very seldom labeled with horseradish peroxidase activity. This strongly suggests that gentamicin treatment impairs the ability of lysosomes to fuse with incoming endocytic vesicles.

Influence of the Binding of Aminoglycosides to Negatively Charged Membranes on Membrane Permeability to Ca^{2+} , Mn^{2+} , Calcein, and Inulin

The ability of lipids to self-assemble into fluid bilayer structures is consistent with one of the major roles played by membranes, that is, their ability to provide a permeability barrier between external and internal environments. An intact permeability barrier to small ions such as Na^+ , K^+ , and H^+ for example, is vital for establishing the electrochemical gradients that give rise to a membrane potential and drive other membrane-mediated transport processes.

Since aminoglycoside affects the lateral phase organization of lipids, which is a membrane property related to permeability, we have investigated the effect of these drugs on the permeability of phosphatidylinositol-containing liposomes toward calcein, Mn^{2+} , Ca^{2+} , and inulin and on the permeability of cultured cells exposed to Ca^{2+} .

Permeability to calcein was studied as described by Weinstein et al. (1977) and Allen and Cleland (1980), based on the dequenching of its fluorescence upon dilution. Thus calcein is encapsulated in liposomes at a high concentration, at which probe-probe interaction results in fluorescence self-quenching. When the fluorophore leaks from the liposome, it is diluted into

a large volume, and its fluorescence increases markedly. Mn^{2+} permeability can be studied by the technique described by Hope et al. (1985). Mn^{2+} quenches the ^{51}P NMR signal of the phospholipids to which it has a direct access, by broadening the resonance signal beyond the detection level. Leakage of Mn^{2+} from the intravesicular space can therefore be monitored by following the increase of the NMR signal due to the dequenching of phospholipids of the internal side of the liposomes. Mn^{2+} is prevented from rebounding to the external layer of phospholipids by the addition of an excess of ethylenediamine tetraacetic acid (EDTA). Ca^{2+} and inulin efflux was monitored by measuring the amount of dialyzable radioactivity of preparations of $^{45}Ca^{2+}$ and [^{125}I]inulin-loaded large unilamellar liposomes exposed to the drugs for up to 24 h.

For cell Ca^{2+} permeability studies, cells were loaded with the acetoxy-methyl ester of Fura-2-(1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzo-furan-5-oxyl]-2-(2-amino-5-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid penta-acetoxy-methyl ester), abbreviated as Fura-2 AM (Gryniewicz et al., 1985), by a 20-min incubation in the dark at room temperature in the presence of 10 μM of the dye. Once inside the J774 macrophages, Fura-2 AM is cleaved in its free pentacarboxylic acid form (Fura-2) by cytosolic esterases, which keeps it trapped intracellularly because of its strongly charged and hydrophilic character (Di Virgilio et al., 1988). The whole excitation spectrum of Fura-2 was determined by scanning the preparation at wavelengths between 300 and 425 nm and recording the emitted light of individual cells at 510 nm. As described by Gryniewicz et al. (1985), the formation of Ca^{2+} -Fura-2 complexes is accompanied by a shift of the maximum of the absorption spectrum from 365 to 335 nm, and the ratios of the emitted intensities observed at the excitation wavelength of 340 and 380 nm allow monitoring of the change of Ca^{2+} concentration in cells.

The results of these investigations are presented in a condensed fashion in Table 1. It is striking that in all four systems, isepamicin gave a strong response, whereas gentamicin was almost completely inactive. Amikacin

TABLE 1. Effect of Aminoglycosides on Membrane Permeability to Ca^{2+} (Liposomes and Cells), Mn^{2+} (Liposomes), and Calcein (Liposomes)

Drug	Permeability			Cells, Ca^{2+}
	Liposomes Ca^{2+}	Liposomes Mn^{2+}	Liposomes Calcein	
Isepamicin	+++	++	+++	++
Amikacin	++	+	++	+
Gentamicin	+	-	+	-

Note. Details of techniques used and quantitative results are described in Van Bambeke et al. (1993).

showed an intermediate behavior. Similar results have been obtained with a marker of higher molecular weight (inulin) (manuscript in preparation).

Although an increase in membrane permeability has already been demonstrated upon the formation of lateral domains in the plane of the membrane toward Na^+ (Papahadjopoulos et al., 1973), water (Carruthers & Melchior, 1983), and nonelectrolytes of molecular weight values up to 900 (van Hoogewest et al., 1984), the role of domain formation in the increase of permeability induced by the aminoglycosides studied is challenged by the observation that isepamicin was among the most potent agents to cause an increase in membrane permeability, whereas it does not induce domain formation in vesicles made of phosphatidylcholine and phosphatidylinositol alone (Schanck et al., 1992b).

Moreover, this increase of membrane permeability induced by aminoglycosides cannot be related to a porogenic, fusogenic, or aggregating action, since the results obtained respectively with melittin, bis(beta-diethylaminoethyl ether) hexestrol, and spermine are totally different. Other hypotheses therefore need to be investigated. Among them, modifications of the degree of lipid acyl chain unsaturation (In't Veld et al., 1992) or accumulation of lipid oxidation products (Curtis et al., 1984; Cirrotti & Thomas, 1984; Ohyashiki et al., 1986) could be interesting.

The results of the conformational analyses of aminoglycoside-phosphatidylinositol mixed monolayers may, however, actually provide a clue for the differences seen among the drugs studied. Thus, as shown here and in Brasseur et al. (1984, 1985a; see also the review by Tilkens et al., 1990), a major difference between gentamicin on the one hand, and isepamicin (and amikacin) on the other hand, is the orientation of the drug in the phospholipid monolayer. Thus, the molecules that adopt an orientation perpendicular to the hydrophobic-hydrophilic interface may set somewhat apart the fatty acid chains, and perhaps dissociate from one another, creating a local condition of disorder, which could cause a potential access to water and ions. This possibility needs to be critically examined by using other agents causing a similar type of disorganization.

RELATIONSHIP BETWEEN INTERACTIONS OF AMINOGLYCOSIDES WITH MEMBRANES AND CELLULAR NECROSIS

The relationship between the interactions of aminoglycosides with phospholipids and in the cellular necrosis these antibiotics are known to elicit in the renal cortex and in the inner ear remains a subject of intense controversy (Lietman, 1985). There are indeed a number of biological processes that can, in principle, be impaired by such interactions, and, accordingly, a large body of literature is available in this respect. Table 2 lists several of them that have been described both *in vivo* and *in vitro*, suggesting therefore that they are of biological significance. For none of them has a causal relationship between the functional alteration and eventual cell death been un-

TABLE 2. Potential Membrane or Metabolic Disturbances Induced In Vivo Through the Binding and Interaction of Aminoglycosides with Phospholipids

Alterations	Proposed mechanisms	References
1. At the level of the pericellular membrane		
Shedding of membrane enzymes and antigens	Binding and membrane disturbance	Mondorf et al. (1978)
Inhibition of phosphatidylinositol-phospholipase C	Binding to phosphatidylinositol	Schwartz et al. (1984)
Inhibition of Na ⁺ /K ⁺ -ATPase	Binding to acidic phospholipids surrounding the enzyme	Williams et al. (1984) Lipsky and Lietman (1980)
2. At the level of the cytosol		
Inhibition of phosphatidylinositol phospholipase C, and of the metabolism of phosphoinositides and related compounds	Binding to phosphatidylinositol Binding to phosphoinositides	Lipsky and Lietman (1982) Schacht (1979)
3. At the level of the mitochondria		
Impairment of oxidative metabolism	Competition with Mg ²⁺	Simmons et al. (1980) Weinberg and Humes (1980a,b) Humes et al. (1982)
Impairment of Ca ²⁺ mitochondrial process	Competition with Ca ²⁺	Sastrasinh et al. (1982) Mela-Riker et al. (1986)
4. At the level of the lysosomes		
Accumulation of undigested phospholipids	Binding to acidic phospholipids	Laurent et al. (1982) Mingeot-Leclercq et al. (1988)
Impairment of lysosome-endosome fusion	?	Giurgea-Marion et al. (1986)
Stimulated exocytosis of lysosomal content	?	Bernard et al. (1987) Josepovitz et al. (1985)
Alteration of lysosomal permeability	?	Powell and Reidenberg (1982, 1983)

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ambiguously demonstrated, however. The reasons for such a failure may be multiple and probably include several of the following:

1. It is uncertain whether some of the major changes demonstrated in vitro really occur in vivo before cell necrosis or whether they only develop after death and during the ensuing drug redistribution from the lysosomes. This is all the more evident for those changes that require a drug concentration that is not achieved at the putative site of action while the cell is still viable. This could apply to cytosolic effects, effects associated with the binding of the drug with the inner face of the pericellular membrane, or effects depending on the interaction of the drugs with mitochondria. This point has been extensively discussed by Tulkens (1986, 1989) and is therefore not reemphasized here.
2. Although many of the changes seen in membrane or membrane-related functions can theoretically lead to cell death, the cell may actually compensate or correct for these changes at least until a certain limit is reached. For instance, the inhibition of Na⁺/K⁺-ATPase is often thought to lead to ionic imbalance, but to what extent this imbalance triggered by aminoglycosides is irreversible and/or not correctable has not been investigated.
3. It is likely that many changes are not lethal until a critical threshold is reached. Little is actually known about the level of such a threshold, but it makes the study of the relationship between primary alterations and cell death particularly difficult. It may also lead to the erroneous conclusion that a change or an early alteration is not a critical one since it occurs in the absence of signs of toxicity.
4. It is perfectly possible that no single change or alteration is important, but that the cell eventually dies because of the simultaneous occurrence of multiple changes. Thus Kaloyanides (1984a, 1984b) suggested that aminoglycoside nephrotoxicity is actually the resulting effect of multiple minor metabolic derangements, which individually are of little vital importance and of dubious significance but which combined or added to each other will jeopardize the ability of the cell to maintain its structural or functional integrity, or defeat its adaptive or reparative capabilities.
5. Finally, there seems to be a major qualitative difference between the massive cell necrosis that occurs under experimental conditions of intoxication (high doses) (Parker et al., 1980) and the more dispersed focal necrosis and apoptosis associated with an extensive proliferation response observed at low, clinically relevant doses (see Cuppage et al., 1977; Houghton et al., 1986; Hollendorff & Gordon, 1980; Laurin et al., 1983; 1988).

Thus, clearly, more experimental studies are needed before mechanistic explanations can be put forward to link membrane alterations induced by aminoglycosides and cell death. In the context of the studies presented earlier, however, it is clear that the lysosomal changes described originally in rat kidney cortex by Kosek et al. (1974) and further analyzed in cultured cells

and in the human kidney (Aubert-Tulkens et al., 1979; De Broe et al., 1984), namely, the lysosomal phospholipidosis, may be good candidates for explaining further cell alterations leading to cell death. It is indeed the earliest abnormality associated with aminoglycoside accumulation within proximal tubular cells, and actually precedes any other sign of cell suffering or injury. This argument, however, has often been used to support the idea that phospholipidosis is not a toxic event per se. Yet it is remarkable that phospholipidosis develops together with apoptoses—not frank necrosis—and significant tubular dedifferentiation (or an increase in the number of poorly differentiated, immature tubular cells) as studied at low, clinically relevant doses (Laurent et al., 1983; Toubeau et al., 1986). The association, however, remains purely circumstantial. Yet, over the last decade, a series of additional converging arguments linking phospholipidosis to cell damage and nephrotoxicity has been presented.

Thus, studies comparing various aminoglycosides have evidenced a direct relationship between phospholipidosis and aminoglycoside nephrotoxicity (Carlier et al., 1983; Tulkens, 1986, 1990). This argument, however, has often been disputed because *in vitro* data cannot be extrapolated to the *in vivo* situation without caution. Thus, the *in vitro* potential of an aminoglycoside to inhibit phospholipid catabolism must be examined within the context of its cellular accumulation and its overall tissue pharmacokinetics for being of predictive value of its toxicity *in vivo*. These parameters indeed will modulate the intrinsic toxicity potential of the drug, and ignoring them will make the extrapolation meaningless. Further indications that phospholipidosis and nephrotoxicity are linked have emerged from the observation that the modulation of phospholipidosis also modulates the toxicity of the aminoglycosides. Thus, it has been reported that the coadministration of anionic polypeptides such as poly-L-aspartic acid with gentamicin or amikacin completely suppresses the histopathological and physiopathological signs of gentamicin nephrotoxicity (Williams & Hottendorf, 1985; Williams et al., 1986; Gilbert et al., 1989; Ramsammy et al., 1989, 1990). At the same time, polyaspartic acid also almost completely prevents the development of the lysosomal phospholipidosis and phospholipiduria induced by gentamicin (Beauchamp et al., 1986, 1990; Ibrahim et al., 1989). As elegantly demonstrated by Kishore et al. (1990a, 1990b, 1992), polyaspartic acid actually acts by binding the aminoglycoside in the lysosomes, thereby preventing it from binding to phospholipids and from inhibiting lysosomal phospholipases and initiating phospholipidosis. Interestingly enough, poly-L-glutamic acid, which fails to protect against phospholipidosis because it does not resist to lysosomal proteases in cells, also fails to prevent development of histopathological and functional signs of aminoglycoside-induced toxicity. Along the same lines, other conditions also known to reduce lysosomal phospholipidosis caused by aminoglycosides, such as once-daily administration (Tulkens, 1991a, 1991b), coadministration with piperacillin

(Carlier et al., 1987), or diabetes mellitus (Vaamonde et al., 1986), also lead to better tolerance of the drugs.

A major problem, however, is that we have no clue as to how phospholipidosis could eventually cause cell death. Lysosomal dilatation per se is not a well-established cause of cell death, as it occurs in many instances of inherited or acquired lysosome storage disorders. Similarly, the presence of undigested phospholipids is a common observation in several cases of phospholipidosis induced by the so-called cationic amphiphiles (see review in Lillmann-Rauch, 1979), which are not associated with early cell death. We have so far failed to detect abnormal phospholipids in the kidney of animals treated with gentamicin. Fibroblasts cultured in the presence of gentamicin show an elevated amount of bis(monooacylglycerol) phosphate, but this phospholipid is also seen in several instances of lysosomal overloading not associated with cell death. Other mechanisms, such as an effect of aminoglycosides on lysosome capacity to fuse and to separate from other membranes, or on lysosome membrane permeability, have therefore been extensively discussed. Membrane fusion and fission play a crucial role in the process of both endocytosis and exocytosis, and their impairment may therefore cause profound alterations of the various digestive, excretory, and secretory activities that are of vital significance in the economy of the cell. The integrity of many cellular organelles and certain features of the functional coordination between cells are also closely linked to this aspect of membrane behavior. The sequential and temporal organization of the membrane fusion reaction plays an important part in the overall balance of cellular activity. Actually, the studies performed by Gurgea-Marion et al. (1986) demonstrate that gentamicin inhibits the fusion of the pinocytic vesicle with lysosomes under clinical conditions of administration. Whether this effect will cause cell death remains, however, unestablished. The reason this hypothesis is presented here with some emphasis is that the actual concentrations of aminoglycosides recorded *in vivo* make it a perfectly plausible mechanism.

Conversely, an alteration of lysosomal membrane permeability by aminoglycoside, although also a plausible hypothesis, has so far received little experimental support. It was reasoned that the presence of drug-lipid complexes could disturb membrane integrity and structure and lead to a loss of the membrane selective permeability to solutes (Lipsky & Lietman, 1982; Hostetler & Hall, 1982; Schwartz et al., 1984; Aramaki & Tsuchiya, 1989). Yet the studies of Van Bambeke et al. (1993) performed *in vitro* and in cultured cells have shown that it is those aminoglycosides with the lowest nephrotoxic potential that induce the larger effect on membrane permeability. Thus there is no simple correlation between this parameter and toxicity (without the possibility of correcting the *in vitro* ranking on the basis of the pharmacokinetic parameters of the drugs studied). We personally consider the studies made with isolated lysosomes to be of little informative value, since

in most cases aminoglycosides were added to the preparation after isolation of the lysosomes rather than being injected to the animal, thus placing the drug not inside but outside the lysosomes, and because we do not know what the fragility of isolated lysosomes demonstrated *in vivo* means in terms of *in vivo* behavior. Actually, the only indication that lysosomes may rupture *in vivo* comes from the scanty reports of images of "destroyed lysosomes" in proximal tubular cells that otherwise look normal during the course of aminoglycoside treatment (Kosek et al., 1974; Tulkens et al., 1984). These images, however, may be of artifactual nature.

In conclusion, we believe that there is now a large body of circumstantial and experimental evidence supporting the concept that aminoglycoside nephrotoxicity is related to lysosomal phospholipidosis, but we also consider that the understanding of the precise mechanism of the cell death caused by these agents remains uncovered so far. Unraveling this mechanism will probably require an improvement in our experimental approaches, as well as a deepening of our knowledge of the cell necrosis process itself and of its triggers. We have, so far, little pertinent information concerning the precise mechanism of the necrosis process in the inner ear induced by aminoglycosides, and therefore of the molecular mechanisms responsible *in fine* for auditory toxicity of these drugs.

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PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL USEFUL IN PREDICTION OF THE INFLUENCE OF SPECIES, DOSE, AND EXPOSURE ROUTE ON PERCHLOROETHYLENE PHARMACOKINETICS

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The ability of a physiologically based pharmacokinetic (PBPK) model to predict the uptake and elimination of perchloroethylene (PCE) in venous blood was evaluated by comparison of model simulations with experimental data for two species, two routes of exposure, and three dosage levels. Unanesthetized male Sprague-Dawley rats and beagle dogs were administered 1, 3, or 10 mg PCE/kg body weight in polyethylene glycol 400 as a single bolus, either by gavage or by intrarterial (ia) injection. Serial blood samples were obtained from a jugular vein cannula for up to 96 h following dosing. The PCE concentrations were analyzed by headspace gas chromatography. For each dose and route of administration, terminal elimination half-lives in rats were shorter than in dogs, and areas under the blood concentration-time curve were smaller in rats than in dogs. Over a 10-fold range of doses, PCE blood levels in the rat were well predicted by the PBPK model following ia administration, and slightly underpredicted following oral administration. The PCE concentrations in dog blood were generally overpredicted, except for fairly precise predictions for the 3 mg/kg oral dose. These studies provide experimental evidence of the utility of the PBPK model for PCE in interspecies, route-to-route, and dose extrapolations.

Knowledge of the influence of different species, dose levels, and routes of administration on the kinetics of environmental chemicals has been considerably enhanced by the use of physiologically based pharmacokinetic (PBPK) models (Clewell & Andersen, 1985; Reitz et al., 1988). Similarities in the anatomy and physiology of mammalian species make interspecies extrapolations of pharmacokinetic data (i.e., animal scale-up or -down) possible (Dedrick, 1973; Boxenbaum, 1984). Pharmacokinetic models developed in one species may be scaled, on the basis of allometric relationships, to allow prediction of chemical concentrations in other species. Model input

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