STUDIES OF A POTENTIAL *IN VITRO* TEST FOR ESTIMATION OF TOXICITY OF AMINOGLYCOSIDE ANTIBIOTICS AND POLYAMINES

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The electrophoretic mobility of phosphatidyl inositol liposomes at pH 7.4, 25°C, was reduced by aminoglycoside antibiotics, neamine and several polyamines in general accordance with the number of amino-groups on each molecule. There was good agreement between the relative position of the tested compounds on the mobility-concentration graph and available information about their relative mammalian toxicities *in vivo*. The slope of the graph for netilmicin was distinctively flat; a comparatively flat dose-response curve for netilmicin has been reported also from *in vivo* studies of nephrotoxicity. Investigation of a homologous series of α,ω straight chain diaminoalkanes revealed that hydrophobicity did not contribute significantly to the observed interaction in this system. L-Lysine showed the weakest effect amongst all compounds tested, supporting the view that the overall positive charge on the molecule was the major determinant of the observed effect. Further structure-activity work is required to confirm whether this '*in vitro*' test is predictive of the toxicity of aminoglycoside antibiotics in man.

The exact sequence of events leading to the expression of mammalian toxicities of aminoglycoside antibiotics is unknown at present. It is uncertain if a single mechanism is responsible for renal, cochlear and vestibular toxicities of these drugs. It seems likely, however, that the selective toxicities of aminoglycosides towards the kidney and the inner ear are at least partly due to the high uptake and retention of the antibiotics by these tissues as compared to other sites in the body[1,2]. Thus, binding at a membrane receptor may be one of the key toxic events. Polycationic drugs bind to biological polyanions such as acid phospholipids contained in mammalian membranes[3]. We have shown[4] that microelectrophoresis was a convenient technique to study the interaction of aminoglycosides with liposomes prepared from acid phospholipids, *e.g.* phosphatidyl inositol. The technique was sufficiently sensitive to detect reduction of electrophoretic mobility of the liposomes at antibiotic concentrations comparable to those found in animal and human studies, even at ionic strength and calcium ion concentrations typically found *in vivo*. The previous results indicated that the attraction between positively charged nitrogen groups on the antibiotics and the negatively charged groups of acidic phospholipids was predominantly responsible for the observed effected. KUNIN[5] suggested that the number of basic sites on aminoglycosides correlated with their binding to tissues and with their toxicity. We decided therefore to include in our study polyamines, a series of structurally related diamines and L-lysine to see whether features other than the overall positive charge played an important role in the observed interaction. Furthermore, we wished to test if the unusual behaviour of netilmicin *in vitro*[6] would be re-confirmed by comparison with a wider group of aminoglycosides, particularly as netilmicin has been reported to have an unusually flat dose-toxicity response curve *in vivo*[7,8].

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Materials and Methods

The antibiotics used were amikacin base (Mead Johnson Laboratories Ltd.), sulfates of dihydrostreptomycin, kanamycin, neomycin and streptomycin (Sigma Chemical Co. Ltd.), ribostamycin sulfate (Morrith Laboratories Ltd.), UK 18892 (butikacin) and UK 31214 (Pfizer Ltd.), tobramycin base (Lilly Research Centre Ltd.), gentamicin and netilmicin sulfates (Schering Corp.) and neamine base (Upjohn Co.).

Spermine tetrahydrochloride; spermidine trihydrochloride; 1,2-diaminoethane dihydrochloride; 1,4-diaminobutane (putrescine) dihydrochloride; 1,5-diaminopentane (cadaverine) dihydrochloride; 1,6-diaminohexane; 1,8-diaminooctane; 1,10-diaminodecane; 1,12-diaminododecane and L-2,6-diaminohexanoic acid (L-lysine) dihydrochloride were purchased from Sigma (London) Chemical Co. Ltd., and phosphatidyl inositol from Lipid Products (South Nutfield, U.K.). All compounds were of the highest grade commercially available, and they were used without further purification.

The experimental procedure was described in detail previously[4]. Briefly, multilamellar phosphatidyl inositol liposomes (0.1 mg lipid/ml) were suspended in tris buffer (pH 7.4, ionic strength 0.092M) containing various concentrations of the test substances. The electrophoretic mobilities of fresh liposomes were determined at 25°C in a Rank micro-electrophoresis apparatus, mark II (Rank Brothers, Bottisham, Cambridge, U.K.) using a flat cell and platinum black electrodes.

Linear regression analysis of electrophoretic mobility against logarithm of molarity ($\geqq$0.02 mM) was performed for each compound. The calculated best straight lines together with maximum standard deviations are shown in Figs. 1 and 2.

Results and Discussion

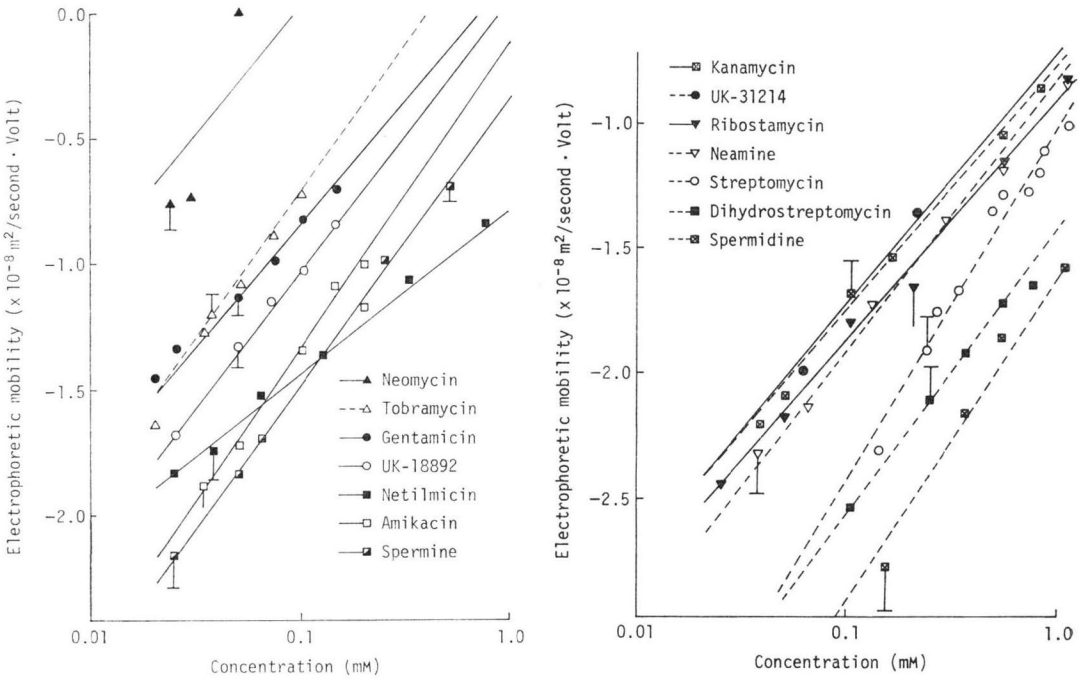
Fig. 1 shows the effect of various concentrations of aminoglycoside antibiotics and polyamines on the electrophoretic mobility of phosphatidyl inositol liposomes. Previously studied antibiotics have been included in the present series in order to test the reproducibility of the method; the results for these antibiotics are similar to those previously reported[4,6]. Batch-to-batch variation of the commercial phosphatidyl inositol may be responsible for some differences in absolute values of mobility between these and previous results. We have evidence that the effect of gentamicin also depends on the batch, presumably caused by the variable composition of gentamicin[9].

With the exception of netilmicin, the order of increasing effect parallels the number of basic nitrogen groups on the aminoglycosides. Neamine, a fragment of neomycin and the polyamines spermidine and spermine with 3 and 4 basic nitrogens, respectively, also conform to this behaviour. The reduction of electrophoretic mobility is very much smaller for compounds with only two positive charges as illustrated in Fig. 2. The mobilities are affected less by diamines than by calcium ions (Fig. 2), or barium and magnesium ions (unpublished results). L-Lysine, a diamino-carboxylic acid with a net charge of +1, shows the weakest effect of all the compounds tested.

There does not appear to be any systematic trend in the effect exerted by the homologous series of α, ω straight chain diaminoalkanes ($C_2 \sim C_{12}$). The hydrocarbon chain length, or the degree of hydrophobicity, would be expected to affect the electrophoretic mobility in a systematic way if either the chains penetrated the lipid bilayer, or the chains projected away from the liposome into the bulk solution. The results indicate, rather, that the chains lie approximately parallel to the surface of liposomes. The absence of a significant hydrophobic effect in this homologous series strengthens the observation[4] that for a variety of polycations used in our studies, the net charge dictates the general position on the mobility-concentration plot for a given compound. Other structural features may give rise to the smaller differences in mobility between substances with the same overall positive charge. The importance of the

Fig. 1. The dependence of electrophoretic mobility of phosphatidyl inositol liposomes (tris buffer, pH 7.4, I=0.092 M, 25°C) on concentration of various polycationic substances.

Results for higher concentrations of neomycin (including U>0), dihydrostreptomycin, streptomycin and spermidine used for regression analysis are not shown. Many of the points represent averages of several independent experiments.

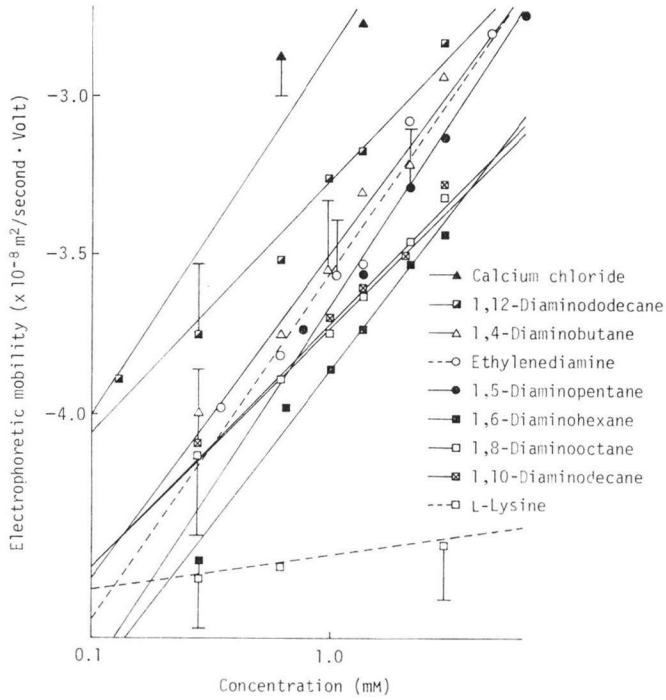


spatial distribution of the positive charges is evidenced by the much greater effect of the bivalent metal cations compared with diamines.

At concentrations above 0.02 mM, the lines for all compounds are approximately parallel, with the important exception of netilmicin whose slope is different and L-lysine which shows a marked non-linear mobility-concentration dependence at molarities comparable to the other compounds. Netilmicin has 5 basic nitrogen groups but at higher concentrations, its mobility line crosses to the region of compounds with only 4 basic nitrogens. We have no explanation for this behaviour at present. UK 31214 is a 1-*N*-dihydroxyisopropyl kanamycin B derivative[10]; the parent compound has 5 basic nitrogens but in UK 31214 the presence of the bulky substituent containing two electron-withdrawing hydroxyl groups makes the 1-amino group much less basic. This can be contrasted with UK 18892 (butikacin) (A. B. RUSSELL, personal communication, Pfizer Central Research Ltd., U. K., 1979) where the substitution of a hydrogen by a hydroxybutylamino group in the 1-N position of kanamycin A[11] appears only to have the effect of adding an extra (fifth) basic amino group to the parent compound because the basicity of the 1-NH group is reduced only slightly by the substituent. Similar explanations were offered for the effect of 1-*N*-substituents in aminoglycosides on their inhibition of the respiratory activity of rat kidney mitochondria[12].

The dearth of clinical studies of the comparative toxicity of many aminoglycosides necessitates the additional use of the findings of toxicological studies in animals for correlations with the *in vitro* properties of these drugs. However, extrapolation of high dose studies in animals to man may not be

Fig. 2. The dependence of electrophoretic mobility of phosphatidyl inositol liposomes (tris buffer, pH 7.4, I=0.092 M, 25°C) on concentration of α,ω diaminoalkanes, L-lysine and calcium chloride.
Results for higher concentrations of Ca^{2+}, 1,5-diaminopentane (cadaverine) and L-lysine used for regression analysis are not shown.



always valid[8]. Bearing in mind these complications, the following comparisons of the mobility-concentration graph (Fig. 1) with toxicity can be made. Evidence from studies in animals and man indicates that tobramycin may be less nephrotoxic than gentamicin[7,13~18]. Our technique does not appear to have sufficient resolution to suggest a significant difference between the effects of these two compounds if, indeed, such a difference exists. Netilmicin exhibits a relatively flat dose-nephrotoxic response curve in rats compared to gentamicin[7,8]. It is not clear yet if the same mechanism is responsible for this behaviour *in vivo* and the markedly flatter slope of the mobility-concentration plot of netilmicin compared to all other tested aminoglycosides. Netilmicin is thought to be less nephrotoxic than gentamicin in animals[7,17] and man[14] and was also found to be less nephrotoxic than tobramycin in animal models[7,19~21], but comparable to it in man[14]. In rat, netilmicin is more nephrotoxic than amikacin at low doses[8], but less nephrotoxic than amikacin or kanamycin at high doses. Our *in vitro* findings are consistent with this behaviour. Furthermore, the position of the lines for neomycin and streptomycin in Fig. 1 reflects correctly the fact that these two compounds represent the two extremes in nephrotoxicity amongst the more common aminoglycosides[7,16].

The relative positions of ribostamycin and neamine also appear to be correct: chronic administration of high doses of ribostamycin caused no nephrotoxicity in rats[22] and very little in guinea pigs[23]. PRICE *et al.*[24] suggested that acute i.v. LD_{50} values in mice could be used as predictors of toxicity in humans. They ranked ribostamycin as having only one-fourth of gentamicin and two-thirds of neamine toxicity. KORNGUTH *et al.*[25] suggested that the inhibition of binding to renal subcellular fractions by polyamines correlated well with their nephrotoxic potential. The polyamines spermine, spermidine,

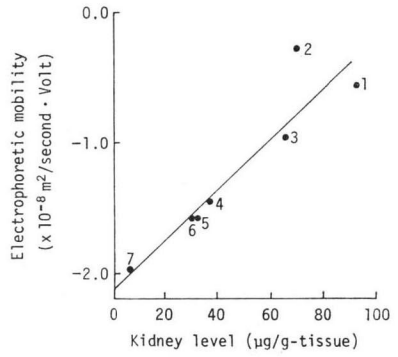
cadaverine and putrescine diminished the binding of gentamicin in the same order as the effect on the electrophoretic mobility of the liposomes (Figs. 1 and 2).

Single-dose studies in dogs and a short-term multiple dose study in the rat[26] indicated that there was a qualitative correlation between renal cortical levels of aminoglycosides and their nephrotoxicity. KOMIYA *et al.*[27] published data on kidney levels of aminoglycosides 24 hours after an intramuscular administration of 20 mg/kg to rats. Fig. 3 was constructed using their data and the electrophoretic mobilities in the presence of 0.2 mM concentrations of aminoglycosides obtained from Fig. 1. The good correlation between the *in vitro* results and the kidney levels ($r^2=0.92$) provides further support for the suggestion[27], that the differences between retention of these drugs in the kidneys could be explained, to a large extent, by the difference in their affinities to renal tissues caused by electrostatic forces. The same forces were proposed to be responsible for the acute toxicity of aminoglycosides[27]. However, it may be pointed out that the correlation between acute LD_{50} values and renal concentration[27] was poorer ($r^2=0.59$) than that shown in Fig. 3. Therefore, there is little justification in any future use of LD_{50} tests as a predictor of human nephrotoxicity.

There have been only a few studies in which the serum levels of aminoglycosides were sufficiently controlled to differentiate between the genuine ototoxic potential and effects secondary to renal damage and subsequent rise of drug levels in the inner ear. Almost imperceptible increases in serum levels could lead to marked drug accumulation in peripheral compartments[28], such as the inner ear fluids. VOLDRICH[29] and STUPP *et al.*[30] demonstrated that the relative cochlear toxicity, neomycin>kanamycin>streptomycin, was in line with the perilymph concentration of these drugs following comparable i.m. doses in guinea-pigs. Similarly, chronically administered systemic tobramycin and amikacin reached lower perilymph levels and were less ototoxic than gentamicin or sisomicin[31]. Systemic administration of ribostamycin caused no[22], or only mild[23], ototoxicity and there was no evidence of accumulation of the drug in the perilymph on repetitive dosing[23]. Although the possibility of concurrent nephrotoxicity in some of these studies renders the rank order of ototoxicity less reliable, nevertheless our microelectrophoresis results are in good agreement with the relative *in vivo* ototoxicity of aminoglycosides. These drugs were administered systemically and their cochlear toxicity appeared to be related to their ability to penetrate and to be retained in the perilymph. On the other hand, the loss of cochlear microphonics during direct perilymphatic perfusion with aminoglycosides produced the following order of ototoxicity[32]: neomycin B= kanamycin B>ribostamycin=gentamicin C_{1a}> kanamycin A≫neamine. The relative ototoxicity of ribostamycin as measured in this test is not in agreement with experimental evidence from systemic administration[22,23]. Several authors[32,33] cited the claim of OWADA[34] that neamine has low ototoxicity but the supporting evidence was never published (K. OWADA, personal communication, 1981). In fact, in contrast to non-ototoxic

Fig. 3. Correlation between the electrophoretic mobility of phosphatidyl inositol liposomes in the presence of 0.2 mM concentrations of aminoglycosides (data obtained from regression lines in Fig. 1) and the kidney levels of these drugs 24 hours after a single intramuscular dose of 20 mg/kg to rats (from Ref. 27).

1=Gentamicin, 2=tobramycin, 3=amikacin, 4=kanamycin, 5=neamine, 6=ribostamycin, 7=streptomycin.



substances, neamine interfered with labelling of polyphosphoinositides in a manner similar to neomycin[35]. Thus, direct perilymphatic perfusion may not be a good predictor of ototoxicity of systemically administered aminoglycosides as it neglects the apparently important pharmacokinetic aspects. Moreover, it is uncertain[36] how well the acute ototoxicity tests relate to the chronic hearing defect associated with the loss of hair cells.

In conclusion, the *in vitro* method described in this paper shows some promise as a predictor of nephrotoxicity and, perhaps even ototoxicity of aminoglycosides and polyamines. It is recommended to use several 'reference' aminoglycosides in comparative studies with new compounds using the same batch of phosphatidyl inositol. This will avoid errors resulting from batch-to-batch variation of the commercial phospholipid and possibly inaccurate calibration of the equipment.

The overall charge on the molecule at physiological conditions, rather than the number of basic amino groups[5], appears to be the major factor in the interaction of aminoglycosides and polyamines with negatively charged phospholipids. The non-electrostatic interactions may play a role which has not been fully exploited in the design of new aminoglycosides. Microelectrophoresis facilitates studies of the effects of salts, ionic strength, pH or temperature on the interaction of aminoglycosides with phospholipids. The use of this technique can therefore help to elucidate the mechanism of this interaction. It seems possible that microelectrophoresis will differentiate correctly between aminoglycosides containing the same number of basic amino groups but showing different levels of toxicity.

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References

1) STUPP, H.; S. RAUCH, H. SOUS, J. P. BRUN & F. LAGLER: Kanamycin dosage and levels in ear and other organs. Arch. Otolaryng. 86: 63~69, 1967
2) LUFT, F. C. & S. A. KLEIT: Renal parenchymal accumulation of aminoglycoside antibiotics in rats. J. Infect. Dis. 130: 656~659, 1974
3) ORSULAKOVA, A.; E. STOCKHORST & J. SCHACHT: Effect of neomycin on phosphoinositide labelling and calcium binding in guinea-pig inner ear tissues *in vivo* and *in vitro*. J. Neurochem. 26: 285~290, 1976
4) ALEXANDER, A. M.; I. GONDA, E. S. HARPUR & J. B. KAYES: Interaction of aminoglycoside antibiotics with phospholipid liposomes studied by microelectrophoresis. J. Antibiotics 32: 504~510, 1979
5) KUNIN, C. M.: Binding of antibiotics to tissue homogenates. J. Infect. Dis. 121: 55~64, 1970
6) HARPUR, E. S.; I. GONDA & R. KINGSTON: Interaction of netilmicin, UK 18,892 (butikacin) and UK 31,214 with phosphatidyl inositol liposomes studied using microelectrophoresis. Toxicol. Lett. S. I. 1: 46, 1980
7) LUFT, F. C.; R. BLOCH, R. S. SLOAN, M. N. YUM, R. COSTELLO & D. R. MAXWELL: Comparative nephrotoxicity of aminoglycoside antibiotics in rats. J. Infect. Dis. 138: 541~545, 1978
8) HOTTENDORF, G. H.; D. BARNETT, L. L. GORDON, E. F. CHRISTENSEN & H. MADISSOD: Nonparallel nephrotoxicity dose-response curves of aminoglycosides. Antimicrob. Agents Chemother. 19: 1024~1028, 1981
9) MICHELSON, P. A.; W. A. MILLER, J. F. WARNER, L. W. AYERS & H. G. BOXENBAUM: Multiple dose pharmacokinetics of gentamicin in man: Evaluation of the Jelliffe nomogram and the adjustment of dosage in patients with renal impairment. *in* "The Effect of Disease States on Drug Pharmacokinetics" *Ed.* L. Z. BENET, American Pharmaceutical Association Academy of Pharmaceutical Sciences, Washington D. C., 1976
10) WISE, R.; J. M. ANDREWS & K. A. BEDFORD: UK 31214: A new aminoglycoside and derivative of kanamycin B. Antimicrob. Agents Chemother. 17: 298~301, 1980
11) COLEMAN, M. V.; M. KINNS & M. J. SEWELL: ^{13}C NMR Studies on 1-*N*-[(*S*)-4-amino-2-hydroxybutyl] kanamycin A (butikacin) and related aminoglycosides. J. Antibiotics 32: 355~360, 1979
12) BENDIRDJIAN, J. P.; B. FOUCHER & J. P. FILLASTRE: Comparative study of the effects of sagamicin, 1N-

HABA-sagamicin, UK-4, Sch 21420, gentamicin and amikacin on the respiration activity of isolated rat kidney mitochondria. J. Antibiotics 33: 1383~1385, 1980

13) FRIMODT-MOLLER, N.; S. MAIGAARD & P. O. MADSEN: Comparative nephrotoxicity among aminoglycosides and beta-lactam antibiotics. Infection 8: 283~289, 1980
14) DAHLAGER, J. I.: The effect of netilmicin and other aminoglycosides on renal function. Scand. J. Infect. Dis. Suppl. 23: 96~102, 1980
15) SCHENTAG, J. J.; M. E. PLAUT & F. B. CERRA: Nephrotoxicity of gentamicin and tobramycin in patients: Pharmacokinetic and clinical studies. Current Chemother. Infect. Dis., Proc. 11th ICC & 19th ICAAC, pp. 614~616, 1980
16) WHELTON, A.; G. G. CARTER, T. J. CRAIG, H. H. BRYANT, D. V. HERBST & W. G. WALKER: Comparison of the intrarenal disposition of tobramycin and gentamicin: Therapeutic and toxicological answers. J. Antimicrob. Chemother. 4 (Suppl. A): 13~22, 1978
17) ORMSBY, A. M.; R. A. PARKER, C. E. PLAMP, III, P. STEVENS, D. C. HOUGHTON, D. N. GILBERT & W. M. BENNETT: Comparison of the nephrotoxic potential of gentamicin, tobramycin and netilmicin in the rat. Current Therap. Res. 25: 335~343, 1979
18) LUFT, C. F. & A. P. EVAN: Comparative effects of tobramycin and gentamicin on glomerular ultrastructure. J. Infect. Dis. 142: 910~914, 1980
19) WEINBERG, E. H.; W. E. FIELD, W. D. GRAY, M. F. KLEIN, G. R. ROBBINS & E. SCHWARTZ: Preclinical toxicological studies of netilmicin. Drug Res. 31 (I): 816~822, 1981
20) MARRE, R.; M. ABRAHAM, H. FREIESLEBEN & K. SACK: Netilmicin and Tobramycin: ver gleichende tierexperimentelle Untersuchung zur Pharmakokinetic, Nieren vertraglichkeit and therapeutishen Effektivitat. Drug Res. 29 (I): 940~945, 1979
21) SZOT, R. J.; G. MCCORMICK, M. CHUNG, B. CHRISTIE, E. WEINBERG & E. SCHWARTZ: Comparative toxicity of netilmicin and tobramycin in dogs. Toxicol. Appl. Pharmacol. 55: 169~178, 1980
22) HARADA, Y.: Ototoxicity of vistamycin in the cochlea of guinea-pigs. Chemotherapy (Tokyo) 20: 255~259, 1972
23) HARPUR, E. S.; F. JABEEN, R. KINGSTON, I. GONDA, K. W. BRAMMER & M. H. GREGORY: Single and multiple dose pharmacokinetics of ribostamycin in serum and perilymph of guinea-pigs in relation to its low toxicity, pp. 31~36, *in* Organ-Directed Toxicity. Chemical Indices and Mechanisms, *Eds.* S. S. BROWN & D. S. DAVIES, Pergamon Press, Oxford, New York, 1981
24) PRICE, K. E.; J. C. GODFREY & H. KAWAGUCHI: Effect of structural modifications on the biological properties of aminoglycosides antibiotics containing 2-deoxystreptamine. pp. 191~307, *in* Advances in Applied Microbiology, *Ed.* D. PERLMAN, Vol. 18, Academic Press, New York, 1974
25) KORNGUTH, M. L.; W. H. BAYER & C. M. KUNIN: Binding of gentamicin to subcellular fractions of rabbit kidney: inhibition by spermine and other polyamines. J. Antimicrob. Chemother. 6: 121~131, 1980
26) NIEMINEN, L.; A. KASANEN, L. KANGAS, E. SAIRIO & M. ANTTILA: Renal accumulation of amikacin, tobramycin and gentamicin in the rat. Experientia 34: 1335, 1978
27) KOMIYA, I.; K. UMEMURA, M. FUJITA, A. KAMIYA, K. OKUMURA & R. HORI: Mechanism of renal distribution of aminoglycoside antibiotics. J. Pharm. Dyn. 3: 299~308, 1980
28) GONDA, I. & E. S. HARPUR: Accumulation in the peripheral compartment of a linear two-compartment open model. J. Pharmacokin. Biopharm. 8: 99~104, 1980
29) VOLDŘICH, L.: Kinetics of streptomycin, kanamycin and neomycin in the inner ear. Acta Oto-laryng. 60: 243~248, 1965
30) STUPP, H.; K. KUPPER, F. LAGLER, H. SOUS & M. HUANTE: Inner ear concentrations and ototoxicity of different antibiotics in local and systemic application. Audiology 12: 350~363, 1973
31) BRUMMETT, R. E.; K. E. FOX, T. W. BENDRICK & D. L. HINES: Ototoxicity of tobramycin, gentamicin, amikacin and sisomicin in the guinea-pig. J. Antimicrob. Chemother. 4 (Suppl. A): 73~83, 1978
32) LODHI, S.; N. D. WEINER, I. MICHIGIAN & J. SCHACHT: Ototoxicity of aminoglycosides correlated with their action on monomolecular films of polyphosphoinositides. Biochem. Pharmac. 29: 597~601, 1980
33) HAWKINS, J. E., Jr.: Biochemical Mechanisms in Hearing and Deafness. *Ed.* M. M. PAPARELLA, p. 323, Charles C. Thomas, Springfield, I. L., 1970
34) OWADA, K.: Experimental studies on the toxicity of kanamycin, its hydrolysed products and neomycin. Chemotherapia 5: 277~293, 1962
35) SCHACHT, J.: Inhibition of neomycin of polyphosphoinositide turnover in subcellular fractions of guinea-pig cerebral cortex *in vitro*. J. Neurochem. 27: 1119~1124, 1976
36) BROWN, D. R. & A. M. FELDMAN: Pharmacology of hearing and ototoxicity. Ann. Rev. Pharmacol. Toxicol. 18: 233~252, 1978