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Uptake of aminoglycoside antibiotics into brush-border membrane vesicles and inhibition of $(Na^+ + K^+)$-ATPase activity of basolateral membrane

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The effects of aminoglycoside antibiotics on plasma membranes were studied using rat renal basolateral and brush-border membrane vesicles. 3′,4′-Dideoxykanamycin was bound to the basolateral membrane and brush-border membrane vesicles. They had a single class of binding sites with nearly the same constant, and the basolateral membrane vesicles had more binding sites than those of the brush-border membrane. Dideoxykanamycin B was transported into the intravesicular space of brush-border membrane vesicles, but not into that of basolateral membrane vesicles. The $(Na^+ + K^+)$-ATPase activity of the plasma membrane fraction prepared from the kidney of rat administered with dideoxykanamycin B intravenously decreased significantly. Aminoglycoside antibiotics entrapped in the basolateral membrane vesicles inhibited $(Na^+ + K^+)$-ATPase activity, but those added to the basolateral membrane vesicles externally failed to do so. The activity of $(Na^+ + K^+)$-ATPase was non-competitively inhibited by gentamicin. It is thus concluded that aminoglycoside antibiotics are taken up into the renal proximal tubular cells across the brush-border membrane and inhibit the $(Na^+ + K^+)$-ATPase activity of basolateral membrane. This inhibition may possibly disrupt the balance of cellular electrolytes, leading to a cellular dysfunction, and consequently to the development of aminoglycoside antibiotics' nephrotoxicity.

Introduction

The aminoglycoside therapy for Gram-negative bacterial infections is known to result frequently in severe nephrotoxicity. Much research in regard to the mechanisms involved and the methods for prevention has been carried out [1–4]. Nephrotoxicity may initially arise from the interactions of aminoglycoside antibiotics with plasma membranes. But the details of the mechanism of aminoglycoside antibiotic accumulation and the progress of nephrotoxicity still remain unclear.

In the present paper, for clarification of the mechanisms of nephrotoxicity, we investigated the interactions between aminoglycoside antibiotics and plasma membranes, brush-border and basolateral membrane vesicles, prepared from rat kidney.

Materials and Methods

Materials. 3′,4′-Dideoxykanamycin B and dideoxy[^{3}H]kanamycin B (66 μCi/mg) were kindly provided by Meiji Seika Kaisha (Tokyo). Gentamycin and amikacin were obtained from Sionogi Pharmaceutical Co., Ltd. (Osaka) and Banyu Pharmaceutical Co., Ltd. (Tokyo), respec-

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tively. [^{3}H]Tetraethylammonium bromide was purchased from New England Nuclear (NET 206-10, U.S.A.). All other reagents were of the best grade available. Male Wistar rats (200–250 g) were purchased from Shizuoka Agricultural Co. (Shizuoka).

Membrane preparations. The brush-border membrane and basolateral membrane vesicles were prepared from the cortex of the isolated kidney by the calcium precipitation method [5] and the Percoll density gradient centrifugation method [6], respectively. The membrane preparations were suspended in 20 mM Tris-Hepes buffer (pH 7.0)/250 mM mannitol.

The sealed or unsealed vesicles of basolateral membrane was discriminated by the method reported by Podevin and Podevin [7], and the membrane sideness of the vesicles (right-side-out and inside-out) was determined by the same method.

In an in vivo experiment, 1 h following the intravenous administration of dideoxykanamycin B (65 mg/kg body wt.), the cortex was homogenized with 10 mM Tris-HCl buffer (pH 7.5)/250 mM sucrose/1 mM EDTA. After centrifugation at $6500 \times g$ for 10 min, the supernatant was further centrifuged at $32000 \times g$ for 20 min and the membrane fraction was obtained as a precipitate.

Uptake of dideoxykanamycin B by membrane vesicles. The uptake of dideoxy[^{3}H]kanamycin B into the brush-border membrane and basolateral membrane vesicles was determined by rapid filtration using a Millipore filter (HA, 0.45 μm) [8,9]. 100 μl of the membrane suspension (20–30 μg protein) was mixed with 100 μl of an incubation medium consisting of 250 mM mannitol and 20 mM Tris-Hepes buffer. To this system were added various concentrations of dideoxy[^{3}H]kanamycin B followed by incubation at 37°C for an appropriate time. 2 ml of the cold incubation medium was then added to the system, followed by rapid filtration through Millipore filters. The membrane vesicles trapped on each filter were washed twice with 2.0 ml of the cold incubation medium. The filters were dried and the radioactivity was counted by a liquid scintillation counter (Aloka 903) using toluene cocktail as the scintillator [10].

To discriminate the mode of binding of dideoxykanamycin B to the membrane surface from the transport into both vesicles, the intravesicular space was changed by pre-incubation (25°C, 30 min) in a 2 mM Tris-Hepes buffer (pH 7.0) at various concentrations of sucrose (250, 375, 500, 750 and 1000 mM). The uptake of dideoxy[^{3}H] kanamycin B and [^{3}H]tetraethylammonium from the incubation medium by the vesicles was determined at 30 min.

Equilibrium gel filtration. ATP (400 μg) dissolved in 10 mM Tris-HCl buffer (pH 7.0) containing 250 μg/ml of gentamycin was loaded on a Bio-Gel P-2 column (1.2×50 cm) pre-equilibrated with the same buffer but containing no ATP. The concentrations of gentamycin and ATP were determined by HPLC [11] and spectrophotometry (260 nm), respectively.

Analytical methods. Protein was determined by the method of Lowry et al. [12] using bovine serum albumin as the standard. The alkaline phosphatase [13], ($Na^+ + K^+$)-ATPase [14], *N*-acetyl β-D-glucosaminidase [15], cytochrome *c* oxidase [16] and glucose-6-phosphatase [17] activities in brush-border membrane, basolateral membrane and the homogenate were measured. Phospholipid was estimated by the method of Chen et al. [18] using phosphatidylcholine (molecular weight assumed to be 800) as the standard.

Results

Identification of brush-border membrane and basolateral membrane vesicles

As shown in Table I, the ratio of alkaline phosphatase activity in the brush-border membrane vesicles was enriched by about 9-times and that of ($Na^+ + K^+$)-ATPase in the basolateral membrane vesicles was enriched of about 17-times that of the homogenate. The cytochrome *c* oxidase, *N*-acetyl β-D-glucosaminidase and glucose-6-phosphatase activities were much lower than those of the homogenate.

The ratio of the sealed basolateral membrane vesicles and their membrane orientation were estimated from the ouabain-sensitive (($Na^+ + K^+$)-ATPase) and insensitive ATPase activities obtained with or without Triton X-100 and ouabain. Since ATP and ouabain hardly diffuse across the membrane, if the basolateral membrane vesicles are sealed right-side-out, both of the ouabain sensitive and insensitive ATPase activities

TABLE I

SPECIFIC ACTIVITY OF MARKER ENZYMES IN HOMOGENATE, BRUSH-BORDER MEMBRANE (BBM) AND BASOLATERAL MEMBRANE (BLM) VESICLES FROM RAT KIDNEY CORTEX

Data are presented as the mean of three experiments.

Enzymes	Homogenate		BBM		BLM	
	specific activity [a]	(ratio)	specific activity	(ratio)	specific activity	(ratio)
Alkaline phosphatase	85.4	(1.0)	794.2	(9.30)	54.6	(0.64)
$(Na^+ + K^+)$-ATPase	22.9	(1.0)	1.4	(0.06)	400.7	(17.5)
N-Acetyl-β-D-glucosaminidase	15.0	(1.0)	1.5	(0.10)	5.7	(0.38)
Glucose-6-phosphatase	57.1	(1.0)	26.3	(0.46)	3.4	(0.06)
Cytochrome *c* oxidase	0.34 [b]	(1.0)	n.d.		n.d.	

[a] Specific activity in nmole substrate/mg protein per min.

[b] Cytochrome *c* oxidase activity was defined as $\Delta A_{550\ nm}$/min.

cannot be measured, and if they are sealed inside-out or an unsealed, both ATPase activities can be measured as the total ATPase. As shown in Fig. 1, the specific activity of $(Na^+ + K^+)$-ATPase is varied with the concentrations of the detergent, and the highest activity was observed at 0.015% of Triton X-100. The activity obtained without Triton X-100 originates from the unsealed sheets, and the latent activity obtained with 0.015% of Triton X-100, which makes the basolateral membrane vesicles permeable to ATP and ouabain, corresponds to the sealed vesicles, inside-out and right-side-out. Thus, the percentage of the sealed vesicles of the basolateral membrane preparation was estimated as 36%.

The membrane sideness of the basolateral membrane vesicles was estimated from the total ATPase activity; 21.5 and 78.5% of the sealed vesicles were right-side-out and inside-out, respectively.

Uptake of dideoxykanamycin B into membrane vesicles

The uptake of dideoxy[^{3}H]kanamycin B by the brush-border membrane and basolateral membrane vesicles increased with the incubation time, reaching a plateau after 30 min at 37°C. The dideoxykanamycin B uptake by both membrane vesicles was examined as a function of the dideoxykanamycin B concentration and showed saturation in the region of high dideoxykanamycin B concentrations (Fig. 2B). A Scatchard plot analysis showed linear regression lines over the concentrations tested (Fig. 2A). The binding parameters are listed in Table II, and indicate that the brush-border membrane and basolateral membrane vesicles have a class of binding sites with essentially the same association constant (K). The

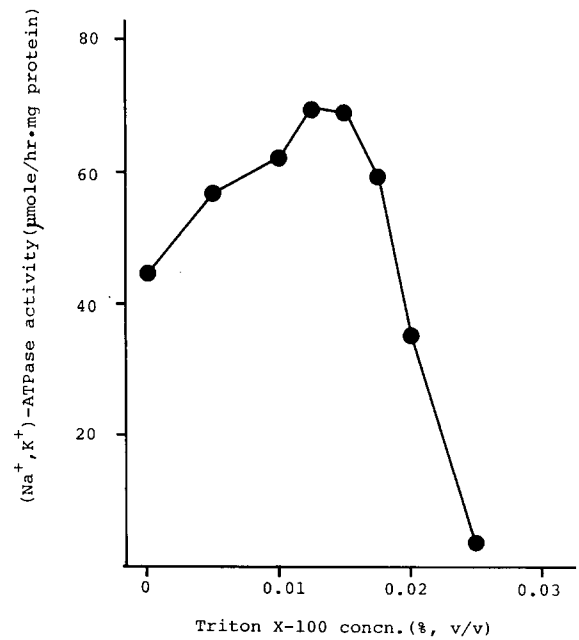

Fig. 1. The concentration dependence of the activation of $(Na^+ + K^+)$-ATPase by Triton X-100.

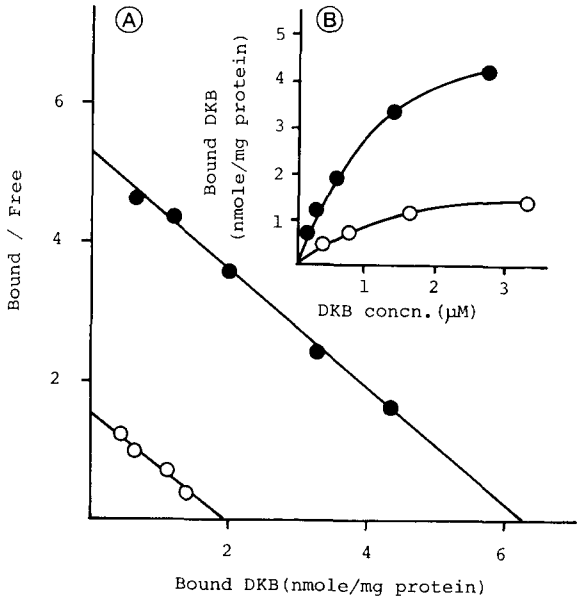

Fig. 2. Scatchard plots (A) and direct plots (B) of dideoxy-[^{3}H]kanamycin B (DKB) binding to rat renal brush-border membrane and basolateral membrane vesicles. ○, brush-border membrane; ●, basolateral membrane.

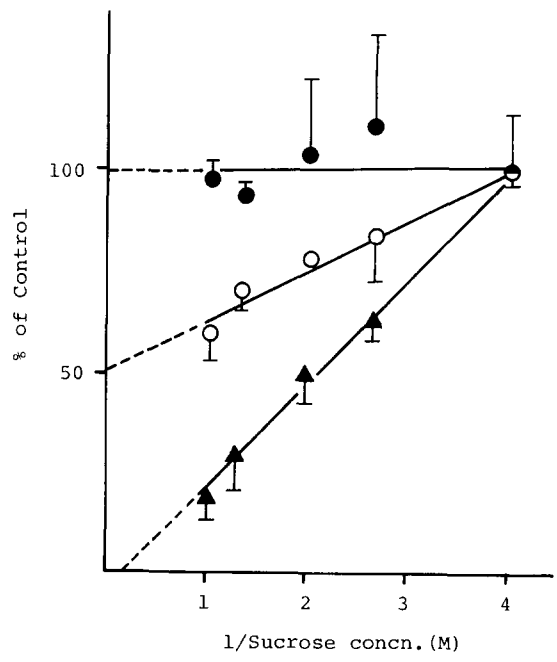

Fig. 3. Effect of osmolarity on the uptake of dideoxy-[^{3}H]kanamycin B by brush-border membrane (○), basolateral membrane (●) vesicles and [^{3}H]tetraethylammonium by basolateral membrane (▲).

basolateral membrane vesicles had from 2- to 3-times as many binding sites as the brush-border membrane vesicles, as indicated by an examination of their phospholipid and protein content, respectively.

The vesicles were pre-incubated in the medium at various osmolarities, and their intravesicular space was noted to change according to the concentration of sucrose. Fig. 3 shows the uptake of dideoxy[^{3}H]kanamycin B by the brush-border membrane vesicles to decrease with increasing sucrose concentrations, but the uptake by basolateral membrane vesicles to remain unchanged, irrespective of the osmolarity. Tetramethylammonium binding to the basolateral membrane vesicles, a control experiment, was correlated inversely with sucrose hypertonicity. Thus, dideoxy-[^{3}H]kanamycin B is transported into the intravesicular space of the brush-border membrane vesicles, but not into that of the basolateral membrane vesicles.

TABLE II

BINDING PARAMETERS OF DIDEOXYKANAMYCIN B AND BRUSH-BORDER MEMBRANE (BBM) OR BASOLATERAL MEMBRANE (BLM) VESICLES

	BBM	BLM
K ($\times 10^6$ M^{-1})	0.97 ± 0.28	1.66 ± 0.60
(nmol/mg protein)	2.30 ± 0.35	6.75 ± 0.46
n (nmol/mol phospholipid)	3.08 ± 0.47	6.42 ± 0.43

Effects of dideoxykanamycin B on alkaline phosphatase and ($Na^+ + K^+$)-ATPase activity in vivo

The membrane fractions prepared from the kidney of the rats administered with dideoxykanamycin B (65 mg/kg body wt., i.v.) were assayed for alkaline phosphatase and ($Na^+ + K^+$)-ATPase activity. The ($Na^+ + K^+$)-ATPase activity of the basolateral membrane fraction (0.5 ± 0.18 μmol/h per mg protein) is inhibited by about 45% compared with the control (0.9 ± 0.09 μmol/h per mg protein), but that the activity of alkaline phosphatase (2.1 ± 0.21 μmol/h per mg protein) is not affected.

Effects of aminoglycosides on ($Na^+ + K^+$)-ATPase activity in basolateral membrane vesicles

Two methods were used to determine the effects of aminoglycosides on the ($Na^+ + K^+$)-ATPase activity. First, in order that aminoglycosides would be entrapped within the intravesicular space, the basolateral membrane vesicles were prepared using 10 mM Tris-HCl buffer (pH 7.5)/250 mM sucrose/1 mM EDTA/1 mM of aminoglycosides as reported by Inui et al. [6]. Secondly, the basolateral membrane vesicles were prepared in the same way except that the aminoglycosides were added externally to the basolateral membrane vesicles in the incubation medium. The ($Na^+ + K^+$)-ATPase activity was found to be significantly inhibited by aminoglycosides in the intravesicular space, but not by externally added aminoglycosides (Table III). Furthermore, the ($Na^+ + K^+$)-ATPase activity was inhibited by gentamycin as its concentration increased. It took about 6 h to prepare the basolateral membrane vesicles with aminoglycosides entrapped in them. It was uncertain that the ($Na^+ + K^+$)-ATPase activity was uninhibited only by the long-term preincubation with aminoglycosides. So, the basolateral membrane vesicles were preincubated with aminoglycosides added externally to the incubation medium at 4°C for 6 h, but the ($Na^+ + K^+$)-ATPase activity was not inhibited (data not shown). Thus, the aminoglycosides present within the basolateral membrane vesicles probably inhibited the ($Na^+ + K^+$)-ATPase activity specifically.

The effect of gentamycin on the ($Na^+ + K^+$)-ATPase activity was determined in the presence of Triton X-100. If aminoglycosides attack ($Na^+ + K^+$)-ATPase only from the interior compartment of the basolateral membrane vesicles, the activity should be inhibited by aminoglycosides when the basolateral membrane vesicles are rendered permeable to ATP by treatment with Triton X-100. The enzyme activity was actually inhibited to 72% of the control by $1 \cdot 10^{-3}$ M gentamycin. This result is consistent with the results listed in Table III.

To determine the type of ($Na^+ + K^+$)-ATPase inhibition by gentamycin, the enzyme activity was

TABLE III

EFFECT OF AMINOGLYCOSIDES ON ($Na^+ + K^+$)-ATPase ACTIVITY OF BASOLATERAL MEMBRANE VESICLES

Aminoglycoside	Concn. (M)	Assay method [a]	Ativity (μmol/mg protein per h)	% of control
Control	0	–	44.5	100
Amikacin	10^{-3}	I	43.4 ± 2.2	97.5
		II	33.4 ± 8.0	75.1
3′,4′-Dideoxy-kanamycin B	10^{-3}	I	42.3 ± 2.7	95.1
		II	27.1 ± 2.2	60.9
Gentamycin	10^{-3}	I	43.6 ± 3.3	98.0
		II	16.7 ± 2.4	37.5
	10^{+4}	I	41.4 ± 3.1	93.0
		II	28.9 ± 1.3	64.9
	10^{-5}	I	40.5 ± 3.8	91.0
		II	37.8 ± 2.6	84.9

[a] I, aminoglycosides were added to basolateral membrane vesicles externally; II, aminoglycosides were entrapped within basolateral membrane vesicles.

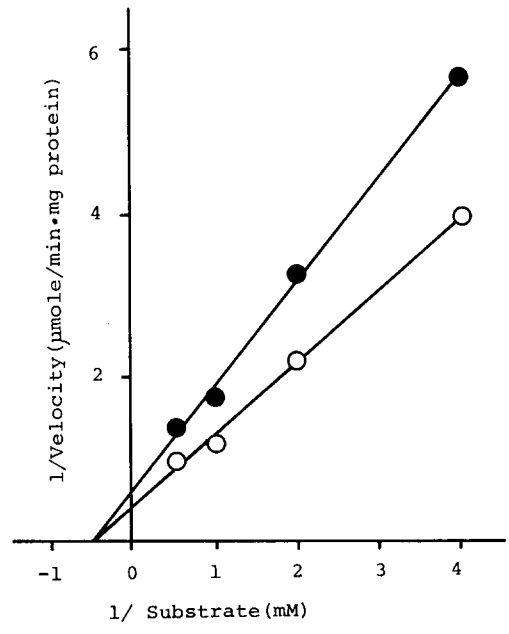

Fig. 4. Lineweaver-Burk plots of the inhibition of ($Na^+ + K^+$)-ATPase in the presence of gentamycin (1 mM) ○, control; ●, gentamycin (1 mM).

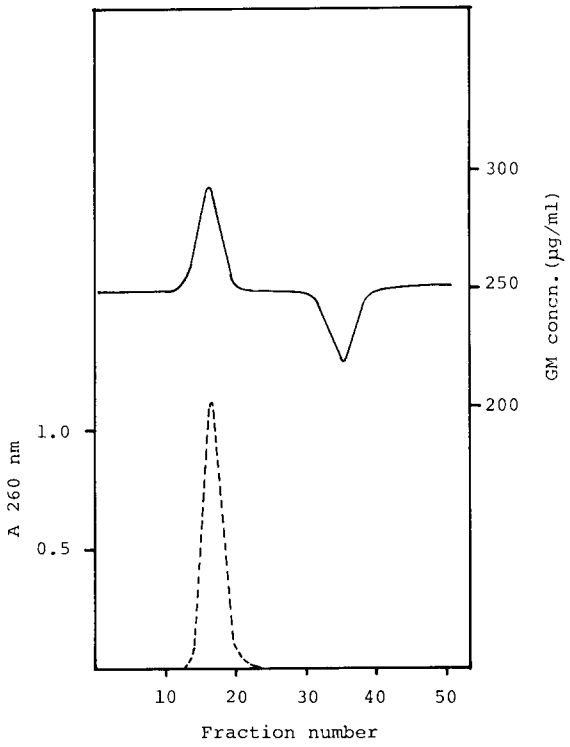

Fig. 5. Equilibrium gel filtration. After the Bio-Gel P-2 column (1.2×50 cm) had been equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing gentamycin (GM) (250 μg/ml), ATP (400 μg) dissolved in the same buffer was applied. The elution was divided into 1.3 ml of fraction. ------, ATP; ———, gentamycin.

measured at various concentrations of the substrate, ATP. The Lineweaver-Burk plots crossed on the abscissa, indicating a non-competitive inhibition (Fig. 4). Thus, gentamycin binds to ($Na^+ + K^+$)-ATPase and/or its substrate, ATP, to form inactive complexes inside the basolateral membrane vesicles.

The binding of gentamycin to ATP was examined by the equilibrium gel filtration method and the elution profiles are shown in Fig. 5. The amount of gentamycin bound to ATP was calculated from the trough of the chromatogram. The complex formed showed a molar ratio of 1 : 1.

Discussion

The uptake of aminoglycosides by and their effects on the brush-border membrane and basolateral membrane vesicles were examined in the present research so as to determine the mechanism of nephrotoxicity. The details of the mechanisms of the interactions between aminoglycosides and plasma membranes cannot be easily clarified by perfusion and in vivo experiments. Isolated membrane vesicles have been widely used to study nephrotoxicity [8,9,19]. Such an approach helps to obviate the complications encountered in studying in vivo or whole cell systems.

In this experiment, the specific activities of the marker enzymes of brush-border membrane, alkaline phosphatase, and of basolateral membrane, ($Na^+ + K^+$)-ATPase, were satisfactorily enriched in each vesicle. Contamination between the brush-border membrane and basolateral membrane vesicles and that of mitochondria, lysosome or microsome should be negligible (Table I).

Based on the present data on dideoxykanamycin B binding, the basolateral membrane vesicles have more binding sites per unit protein content than brush-border membrane (Table II). This is consistent with the results of Williams et al. [20] with gentamycin. Furthermore, Sastrasinh et al. [21] reported aminoglycosides to bind to brush-border membrane with high affinity and this binding to be due to the charge interaction between cationic aminoglycosides and anionic phospholipids, particularly phosphatidylinositol and its derivatives. The phospholipid concentrations of brush-border membrane and basolateral membrane in the present experiment were 0.75 and 1.05 μmol phospholipid/protein, respectively. The number of binding sites of basolateral membrane per phospholipid was twice that of brush-border membrane (Table II). The composition of phospholipids in either brush-border membrane or basolateral membrane of the rat kidney cortex has been reported by Hise et al. [22], and the phosphatidylinositol concentration in basolateral membrane is 1.5-times that in brush-border membrane. Thus, the difference in the phosphatidylinositol concentration between basolateral membrane and brush-border membrane reflects for the most part the number of the binding sites of aminoglycosides. But the difference in the number of binding sites between brush-border membrane and basolateral membrane cannot be explained completely only on the basis of the phosphatidylinositol concentration. A detailed analysis of the phospholipid

compositions in brush-border membrane and basolateral membrane may possible provide the clarification of this point and should thus be carried out.

To determine whether the uptake of dideoxy-[^{3}H]kanamycin B reflect the binding to the membrane or the binding followed by its transport into the intravesicular space, the uptake experiment was carried out at various osmolarities of the incubation medium. With increasing medium osmolarity, the intravesicular space should decrease, and if dideoxykanamycin B is transported into the intravesicular space to a certain concentration, the total uptake value (sum of binding and transport) of dideoxy[^{3}H]kanamycin B should decrease. In the brush-border membrane vesicles, the total uptake value of dideoxy[^{3}H]kanamycin B decreased with increasing osmolarity, but in the basolateral membrane vesicles, the value hardly changed, regardless of the osmolarity (Fig. 3). The vesicles obtained by infinite medium osmolarity have no intravesicular space theoretically, and the extraporated value shows only the binding to the vesicle surfaces. Tetraethylammonium is known to be transported to basolateral membrane vesicles but not bind to its surface [23]. In this experiment, the regression line obtained from tetraethylammonium was extrapolated to the origin and showed the integrity of the basolateral membrane vesicles. The extrapolated value of 50% of the brush-border membrane vesicles shows that half of dideoxykanamycin B taken up at the isotonic osmolarity is transported into the intravesicular space; that is, half of the dideoxykanamycin B taken up by brush-border membrane vesicles binds to the vesicle surface and the other half is transported to the intravesicular space. These findings are compartible with the results of Silverblatt et al. [24] and Wedeen et al. [25] on the basis of the autoradiographic and immunofluorescent microscopic studies, in which gentamycin accumulates into proximal tubular cells by pinocytosis through brush-border membrane. But, if dideoxykanamycin B is assumed to be transported into the intravesicular space of brush-border membrane only by the passive diffusion, more than 95% of dideoxykanamycin B is bound to brush-border membrane and only 5% is transported into the intravesicular space (Fig. 2), because it is well known that the intravesicular volume varies from 1 to 3 μl/mg protein. On the other hand, Saitoh et al. reported that gentamycin was energy-dependently incorporated into the LLC-PK cells from pig kidney [26]. That dideoxykanamycin B is transported into brush-border membrane vesicles but not into basolateral membrane indicates that it accumulates possibly within renal proximal tubular cells from the luminal side through the brush-border membrane, but not from the blood vessel side through the basolateral membrane.

Williams et al. [20,27] reported the ($Na^+ + K^+$)-ATPase activity of rat kidney to be inhibited by gentamycin in vivo and in perfused experiments. In the plasma membrane fraction prepared from the kidney of rats administered with dideoxykanamycin B, the ($Na^+ + K^+$)-ATPase activity was noted to decrease. The ratio of the sealed basolateral membrane vesicles (36%) was compatible with the report by Boumendil-Podevin and Podevin [7].

In the basolateral membrane vesicles, the ($Na^+ + K^+$)-ATPase activity was inhibited when aminoglycosides were entrapped within the intravesicular space (Table III). Inhibition of the ($Na^+ + K^+$)-ATPase activity was also detected by the external addition of gentamycin to the basolateral membrane vesicles which had been made leaky by Triton X-100 treatment. On the other hand, when aminoglycosides were added externally, no enzyme inhibition occurred, since the aminoglycosides could not be transported into the basolateral membrane vesicles. Inhibition of the ($Na^+ + K^+$)-ATPase activity by three kinds of aminoglycoside was comparable to that of the aminoglycosides' nephrotoxicity in vivo, i.e., gentamycin > dideoxykanamycin B > amikacin [28,29].

We have recently demonstrated the binding of dideoxykanamycin B to ATP by equilibrium gel-filtration and the fluorescence quenching method [30]. A gentamycin and ATP complex at a molar ratio of 1 : 1 was also confirmed (Fig. 5). The binding of aminoglycosides to ATP may thus be involved in the mechanism of the ($Na^+ + K^+$)-ATPase activity inhibition. The decrease in the ($Na^+ + K^+$)-ATPase activity may possibly bring about an imbalance in the cellular electrolytes, and consequently cell dysfunction.

Based on the findings of the present research, the inhibition of the $(Na^+ + K^+)$-ATPase activity on the basolateral membrane is concluded to be closely related to aminoglycoside-induced nephrotoxicity.

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