GENTAMICIN BINDING TO BRUSH BORDER AND BASOLATERAL MEMBRANES ISOLATED FROM RAT KIDNEY CORTEX

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The interaction of gentamicin with renal cortex of rats has been studied in vitro by means of a binding assay to brush border and basolateral membranes. Gentamicin specifically bound to plasma membrane fractions, compared to other subcellular fractions. Gentamicin binding to brush border and basolateral membranes was markedly inhibited by polycations such as spermine, and was slightly inhibited by high concentrations of tetraethylammonium. The treatment of phospholipase A2 to both types of membranes increased gentamicin binding, although the treatments by proteolytic enzymes and sulphhydryl reagent did not affect the binding. Gentamicin binding was increased in the brush border membranes treated with acidic phospholipids, whereas it was decreased in the membranes treated with calcium. Judging from the determination of membrane surface charge by metachromasy of cationic dye, basolateral membranes seemed to contain more anionic sites than brush border membranes. The alterations of gentamicin binding described above correlated with the changes of anionic charge on the membranes, indicating a charge interaction between gentamicin and anionic binding sites on the membranes. The addition of other aminoglycoside antibiotics to the incubation mixture induced significant reductions in the binding of gentamicin in the order of aminoglycosides according to their positive charge. The present results suggest that the characteristics of gentamicin binding to brush border and to basolateral membranes are essentially similar, and therefore the renal accumulation of gentamicin may be regulated by the transport of gentamicin across both plasma membranes.

Keywords — aminoglycoside; gentamicin binding; brush border membrane; basolateral membrane; proximal tubule; renal cortex; nephrotoxicity

INTRODUCTION

Aminoglycoside antibiotics are widely used in the treatment of Gram-negative infections, but a limiting factor in the clinical use of aminoglycoside antibiotics is their nephrotoxicity. The aminoglycoside nephrotoxicity appears to be related to the transport and intracellular accumulation of these drugs by renal proximal tubular cells. Understanding the mechanism of cellular accumulation of gentamicin may provide an approach to reduce nephrotoxicity, as well as an insight into fundamental cellular transport processes. The available data suggest that aminoglycosides are taken up into proximal tubular cells across the luminal and basolateral membranes, although the former route of entry is considered to be predominant. The studies of isolated perfused kidney,4) microinjection5–7) and autoradiography8–10) have implicated that aminoglycosides undergo a multistep uptake process at the apical side of renal tubular cells involving binding to anionic sites on brush border membranes, endocytotic uptake and transfer to lysosomes. On the other hand, uptake of aminoglycosides across the basolateral membranes has been inferred from in vitro studies using the renal cortical slice technique11) and the tubule suspension technique.12) Recent studies provide evidence that aminoglycosides may also undergo transtubular secretion.7,13) Uptake via the basolateral membranes of the proximal tubular cells, although considered as a minor route of ami-
noglycoside uptake, may contribute somewhat to the renal cortical accumulation of these compounds. Thus, the renal handling of aminoglycosides remains poorly characterized. The initial interaction of aminoglycosides with renal tubular cells occurs at the plasma membranes. Therefore, in order to obtain more precise information about the mechanisms of aminoglycoside accumulation in renal cortex, we have studied gentamicin binding to brush border and basolateral membranes isolated from rat kidney cortex.

**MATERIALS AND METHODS**

*Materials* — Tobramycin (Shionogi & Co., Osaka, Japan) and amikacin sulfate (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) were kindly supplied. Gentamicin sulfate, neomycin sulfate, kanamycin sulfate, trypsin (from bovine pancreas), papain (from papaya latex type III), phospholipase A₂ (from *Naja naja* venom) and phospholipase C (from *C. perfringens* type I) were obtained from Sigma Chemical Co. (St. Louis, MO). Spermine tetrahydrochloride and tetraethylammonium chloride, p-chloromercuribenzoic acid sodium salt, and *N*-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (Hepes) were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). All other chemicals used for the experiment were of the highest purity available.

*Isolation of Brush Border and Basolateral Membranes* — As reported previously,14-16 brush border and basolateral membranes were isolated from the renal cortex of male Wistar albino rats (190—230 g) by a method of calcium precipitation and Percoll density gradient centrifugation, respectively. All steps were performed on ice or at 4 °C. Usually, brush border and basolateral membranes were suspended in 2 mM Hepes-Tris buffer, pH 7.5, and were used for binding studies on the same day.

In some experiments, brush border and basolateral membranes were pretreated with various enzymes and phospholipids. The membranes were incubated at 37 °C with either trypsin (0.02 mg/mg protein), papain (0.1 U/mg protein), phospholipase A₂ (5 U/mg protein) or phospholipase C (5 U/mg protein) for 30 min. In the preparation of phospholipid-treated membranes, the membranes were incubated at 25 °C with phospholipids (approx. 400 nmol/mg protein) for 30 min. Then, the suspension was centrifuged at 40000 *g* for 30 min. The pellet was suspended in 20 ml of 2 mM Hepes-Tris buffer, pH 7.5 and washed. The centrifugation and washing procedures were repeated. The final pellet was resuspended in 2 mM Hepes-Tris buffer, pH 7.5.

*Binding Studies* — In a routine assay, 200 μl of membrane suspension (250—350 μg protein) was incubated at 4 °C for 10 min with gentamicin (200 μl) in 2 mM Hepes-Tris buffer, pH 7.5. After incubation, the ligand bound to the membranes was separated from the free ligand by centrifugation at 45000 *g* for 30 min at 4 °C. After the supernatant was completely removed, gentamicin in the pellet was extracted with 200 μl of distilled water, and deproteinized with 200 μl of 5% trichloroacetic acid. After centrifugation at 16000 *g* for 20 min, the supernatant was used for the determination of gentamicin.

*Measurement of Charge Density on Brush Border and Basolateral Membranes* — According to the method of Nakagaki et al.,17 the partitioning of methylene blue, a positively charged metachromatic dye, between the membrane phase and the bulk aqueous phase was measured by using visible spectroscopy, and the charge density of the membranes was estimated. Briefly, a membrane suspension (20—120 μl) was added to 20 μM methylene blue solution (3 ml) in 2 mM Hepes-Tris buffer, pH 7.5, and the absorbance at 664 nm was determined or the spectrum between 600 and 700 nm was recorded in a Hitachi spectrophotometer model 200-20. Titrations measurements were carried out by adding equal amounts of protein to the reference and the sample cuvettes. In some experiments, acridine orange (λ=492 nm) was used instead of methylene blue.18

*Analytical Methods* — Gentamicin was determined by a method of substrate-labeled fluores-
cent immunoassay\textsuperscript{19} using Ames TDA gentamicin assay kit (Miles-Sankyo Co. Ltd., Tokyo, Japan). Briefly, 50 \mu l of standard or sample (5 – 25 \mu M) was added to 2.5 ml of dilution buffer. Into each cuvette was added 1.5 ml of diluted antibody/enzyme reagent, followed by 50 \mu l of diluted standard or sample and by 50 \mu l of fluorogenic drug reagent. After the 20 min incubation, fluorescence was measured at 400 nm excitation wavelength and 450 nm emission wavelength using a Shimadzu spectrofluorophotometer RF-510. Protein was determined, after precipitation with ice-cold 10\% (w/v) trichloroacetic acid, by the method of Lowry et al.\textsuperscript{20} with bovine serum albumin as the standard.

RESULTS

Gentamicin Binding to Subcellular Fractions

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig1.png}
\caption{Gentamicin Binding to Subcellular Fractions from Renal Cortex}
\textbf{The subcellular fractions (200 \mu l) were incubated at 4 \degree C for 10 min with 200 \mu l of 1 mM gentamicin in the presence (hatched column) or absence (open column) of neomycin. The reaction mixture was centrifuged at 45000 \textbf{g} for 30 min at 4 \degree C to separate the free ligand. The final concentration for gentamicin and neomycin were 0.5 and 10 mM, respectively. HOM, homogenate; P1, pellet 1; P2, pellet 2; P3, pellet 3; CPM, crude plasma membranes; Fr II, fraction II (basolateral membranes); Fr III, fraction III; Fr IV, fraction IV (brush border membranes); Fr V, fraction V, as described previously.\textsuperscript{16} Each column represents the mean \pm S.E. of two experiments performed in duplicate determinations.}
\end{figure}

In order to obtain information concerning the interaction of gentamicin with renal cortex, we have studied gentamicin binding to subcellular fractions which were prepared from renal cortex by a method described previously.\textsuperscript{14,16} As shown in Fig. 1, there was a strong binding of gentamicin to plasma membrane fractions such as fractions II (basolateral membranes), III and IV (basolateral and brush border membranes), and the bindings were specifically inhibited by the presence of neomycin (10 mM). The specific binding of gentamicin could be estimated by the subtraction of the amount of gentamicin binding in the presence of neomycin (nonspecific binding).

\textit{Gentamicin Binding to Brush Border and Basolateral Membranes}

The binding of gentamicin to brush border membranes as a function of time is shown in Fig. 2. Under the present experimental condition, the binding was found to be very rapid and thus the incubation period for all subsequent binding studies was 10 min.

Fig. 3 shows the effect of neomycin, spermine

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{Effect of Incubation Time on Gentamicin Binding to Brush Border Membranes}
\textbf{The membranes (200 \mu l) were incubated at 4 \degree C with 200 \mu l of 0.4 mM gentamicin in 2 mM Hepes–Tris buffer (pH 7.5). At the indicated time points, the reaction mixture was centrifuged at 45000 \textbf{g} for 30 min at 4 \degree C to separate the free ligand. The final concentration of gentamicin was 0.2 mM. Each point represents the mean \pm S.E. of four determinations from a typical experiment.}
\end{figure}
and tetraethylammonium at various concentrations on gentamicin (0.2 mM) binding to brush border and basolateral membranes. With both membranes, polycations such as neomycin and spermine markedly inhibited gentamicin binding, showing a plateau beginning at a concentration of 2 mM. On the basis of these data, the specific binding of gentamicin at 0.2 mM was approximately 90% of the total binding. On the other hand, tetraethylammonium slightly inhibited gentamicin binding but only at a high concentration of 20 mM. These inhibitory effects were similarly obtained with brush border and basolateral membranes.

**FIG. 3. Effect of Neomycin, Spermine and Tetraethylammonium on Gentamicin Binding to Brush Border (A) and Basolateral Membranes (B)**

The membranes were incubated at 4 °C for 10 min with 0.2 mM gentamicin in the presence of neomycin (●), spermine (△) or tetraethylammonium (○) shown in the abscissa. Each column represents the mean±S.E. of three experiments performed in 2—6 determinations.

**FIG. 4. Scatchard Plots of Gentamicin Binding to Brush Border (A) and Basolateral Membranes (B)**

Each point represents the mean value of 2—3 determinations from a typical experiment.
Gentamicin bindings to brush border and basolateral membranes were also measured at the various concentrations of gentamicin (Fig. 4). Although the Scatchard plots of gentamicin bindings (total) were nonlinear, the deviation from the straight line at low values of bound/free might be explained by the nonspecific binding rather than by the presence of a second binding site. Nonlinear curve fitting provided no evidence for the deviation from a single-site model. Apparent dissociation constant ($K_d$) and the number of binding sites ($n$) are summarized in Table I.

In order to characterize the binding site of gentamicin, brush border membranes were treated with various membrane-modifying reagents and the treated membranes were tested for binding of gentamicin. As shown in Table II, treatments by proteolytic enzymes, trypsin and papain, and the sulphydryl reagent, p-chloromercuribenzoate did not affect gentamicin binding to brush border membranes. On the other hand, treatment by phospholipase $A_2$ greatly increased gentamicin binding. Basolateral membranes treated with these reagents also showed a similar change for gentamicin binding.

Since aminoglycosides are polybasic due to their amino side chains and thus are polycationic at physiological pH, it was thought that acidic sites of the plasma membranes may be targets for a charge interaction with aminoglycosides. In this regard, the electrical potential of the membranes was estimated according to the method of Nakagaki et al.,$^{17}$ who measured the

| TABLE I. Binding Parameters of Gentamicin Binding to Brush Border and Basolateral Membranes |
|---------------------------------|-----------------|-----------------|
|                                | Brush border    | Basolateral     |
| $K_d$ ($\mu$M)                 | 10 ±3           | 22 ±15          | N.S.          |
| $n$ (nmol/mg protein)          | 40 ±6           | 91 ±17          | $p < 0.025$   |

Analysis of the binding data was performed by using the SALS computer program.$^{28}$ Each value represents the mean ± S.E. of five experiments for brush border and four experiments for basolateral membranes.

<table>
<thead>
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<th>TABLE II. Effect of Treatments with Various Reagents on Gentamicin Binding to Brush Border (A) and Basolateral Membranes (B)</th>
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<tr>
<td>Treatment</td>
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<tr>
<td>(A) Brush border membranes</td>
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<tr>
<td>Trypsin, 0.02 mg/mg protein</td>
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<tr>
<td>Papain, 0.1 U/mg protein</td>
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<tr>
<td>p-Chloromercuribenzoate, 0.1 mM</td>
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<tr>
<td>Phospholipase $A_2$, 5 U/mg protein</td>
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<tr>
<td>(B) Basolateral membranes</td>
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<tr>
<td>p-Chloromercuribenzoate, 0.1 mM</td>
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<tr>
<td>Phospholipase $A_2$, 5 U/mg protein</td>
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</table>

The membranes were pretreated with trypsin, papain or phospholipase $A_2$ for 30 min at 37 °C, and then were used for the experiments of gentamicin binding. In the case of p-chloromercuribenzoate, gentamicin binding was measured in the presence of the reagent as described in the text. The final concentration of gentamicin was 0.2 mM. Each value represents the mean ± S.E. of 4–5 determinations from a typical experiment. $^a$) $p < 0.001$, when compared with control value.
partition of methylene blue, a positively charged
metachromatic dye, between negatively charged
phospholipid membranes and the bulk aqueous
phase. The dye was concentrated in the negative-

FIG. 5. Determination of Surface Charge on Brush Border and Basolateral Membranes by Metachromasy
of Methylene Blue
Brush border membranes (○); basolateral membranes (●). (A) Ratio of absorbance (A/A₀) as a function
of membrane protein. Each point represents a single determination. (B) Variation of (N₀M)¹/₂/(N₀ − Nₐ)
as a function of membrane protein. The slopes differ significantly (p< 0.001). A and A₀, absorbance with and
without membranes; N₀ and Nₐ, moles of cationic dye molecule in total and in dimeric form; M, membrane protein amount (mg).

FIG. 6. Effect of Phospholipase Treatment on Gentamicin Binding (A) and Surface Charge (B) of Brush Border Membranes
Brush border membranes were pretreated at 37 °C with phospholipases for 30 min as described for
Table II. (A) Gentamicin binding at 0.2 mM was determined in the presence (hatched column) or absence
(open column) of 4 mM spermine. PLase A₀, phospholipase A₀ treated; PLase C, phospholipase C treated.
Each column represents the mean±S.E. of 3−5 determinations from a typical experiment. a) p< 0.01; b) p< 0.001, when compared with control value. (B) Surface charge was determined as
described for Fig. 5. Each point represents a single determination. The slopes of treated membranes differ
significantly from control (p< 0.001). Control (○); phospholipase A₀ treated (●); phospholipase C
treated (△).
FIG. 7. Effect of Phospholipid Treatment on Gentamicin Binding (A) and Surface Charge (B) of Brush Border Membranes

Brush border membranes were pretreated at 25 °C with phospholipids (approx. 400 nmol/mg protein) for 30 min, and then used for the experiments. (A) Gentamicin binding at 0.2 mM was determined in the presence (hatched column) or absence (open column) of 4 mM spermine. PC, phosphatidylcholine treated; PA, phosphatidic acid treated; PI, phosphatidylinositol treated. Each column represents the mean±S.E. of 3–5 determinations from a typical experiment. a) p<0.01; b) p<0.001, when compared with control value. (B) Surface charge was determined as described for Fig. 5. Each point represents a single determination. The slopes of phosphatidic acid- and phosphatidylinositol-treated membranes differ significantly from control (p<0.001). Control (○); phosphatidylcholine treated (▲); phosphatidic acid treated (●); phosphatidylinositol treated (△).

FIG. 8. Effect of Calcium Addition on Gentamicin Binding to Brush Border Membranes

(A) Gentamicin binding at 0.2 mM was determined in the presence of CaCl₂ as described in Fig. 3. Each point represents the mean±S.E. of 4–5 determinations from a typical experiment. (B) Gentamicin binding at concentrations between 0.1 and 10 mM was determined in the presence (●) or the absence (○) of 0.5 mM CaCl₂. Each point represents the mean value of three determinations from a typical experiment.
ly charged membrane phase and formed dimers, resulting in a reduction of absorbance. Fig. 5A shows the ratios of absorbance with and without membranes (A/Ao) at 664 nm as a function of the membrane protein. The decrease in A/Ao with increasing membrane protein is considered to be due to the partitioning of the positively charged methylene blue molecules into the membrane phase and the formation of dimers. In Fig. 5B, the values of \((N_d M)^{1/2}/(N_o-N_d)\) are plotted against the total protein of the membranes. As the slopes of these plots, \(K\), increase with the negative surface charge of the membrane,\(^{17}\) it appears that the basolateral membranes contain more negative charge than the brush border membranes.

**Charge Interaction between Gentamicin and Brush Border Membranes**

It is conceivable that gentamicin binding to brush border membranes is a consequence of the interaction of polycations with intrinsic anions on the cell surface. Therefore, in order to analyze the increase of gentamicin binding by the treatment of phospholipase, we have estimated the relationship between the alteration of membrane charge and gentamicin binding. As shown in Fig. 6, the treatment of phospholipase \(A_2\) and phospholipase \(C\) increased the gentamicin binding and the negative charge of brush border membranes.

In addition, as is evident from Fig. 7A, gentamicin bindings increased in membranes exposed to acidic phospholipids such as phosphatidylinositol and phosphatidic acid. However, the membranes incubated with phosphatidylcholine, a neutral phospholipid, had no effect on gentamicin binding. Fig. 7B shows that the incubation of brush border membranes with phosphatidic acid and phosphatidylinositol also increased the negative charge of the membranes, whereas incubation with phosphatidylcholine did not.

Lüllmann and Vollmer\(^{21}\) have reported the ability of aminoglycosides to compete with and to displace calcium from acidic phospholipids. As shown in Fig. 8A, the increasing concentrations of calcium decreased gentamicin binding to brush border membranes. Scatchard plots (Fig. 8B) of the inhibitory effect demonstrated that calcium was a competitive inhibitor to gentamicin binding. Thus, calcium would act to neutralize the negative charge of the membranes and thereby decrease the binding of the cationic gentamicin to the membranes.

**Effect of Various Aminoglycosides on Gentamicin Binding**

The effect of several aminoglycosides on gentamicin binding to brush border membranes is summarized in Table III. The addition of other aminoglycoside antibiotics to the incubation mixture at the same concentration of gentamicin induced significant reductions in the binding of gentamicin with the following order of potency: neomycin > tobramycin > amikacin > kanamycin.

<table>
<thead>
<tr>
<th>TABLE III. Effect of Various Aminoglycosides on Gentamicin Binding to Brush Border Membranes</th>
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<tbody>
<tr>
<td>Gentamicin binding</td>
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<tr>
<td>(nmol/mg protein)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Kanamycin</td>
</tr>
<tr>
<td>Amikacin</td>
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<tr>
<td>Tobramycin</td>
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<tr>
<td>Neomycin</td>
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The membranes were incubated for 10 min with 0.2 mM gentamicin in the presence of aminoglycosides (0.2 mM). Each value represents the mean ± S.E. of four determinations from a typical experiment. \(^a\) \(p < 0.001\), when compared with control value.
ycin. This cascade would appear to coincide with the ranking of aminoglycosides according to positive charge at physiological pH \( ^{22} \) and to bear a similarity to the relative nephrotoxicity potentials of these drugs.\(^ {22,23} \)

**DISCUSSION**

The pathogenesis of aminoglycoside nephrotoxicity is generally thought to be related to the accumulation of the drug within the proximal tubular cells. The available data indicate that aminoglycosides enter into the proximal tubular cells across the brush border and basolateral membranes.\(^ {4-13} \) Since the first step in the membrane transport of gentamicin appears to involve binding of the drug to plasma membranes, we have concentrated our efforts on the examination of the characteristics of gentamicin binding to brush border and basolateral membranes.

Josepovitz et al.\(^ {6} \) have reported the inhibition of gentamicin uptake in rat renal cortex *in vivo* by simultaneously infusing other aminoglycosides and organic polycations such as spermine. In the present study, spermine markedly inhibited gentamicin binding to brush border and basolateral membranes, whereas tetraethylammonium, an organic cation, was found to only slightly bind these membranes. Therefore, aminoglycosides may share a common transport system with polyamines rather than with organic cations. The potency of spermine as an inhibitor of gentamicin binding was similar to that of neomycin.

The number of binding sites for gentamicin in basolateral membranes was greater than in brush border membranes, although the affinity to both membranes was not significantly different. Scatchard analysis of gentamicin binding to brush border membranes has been reported by Just and Habermann,\(^ {24} \) Lipsky *et al.*\(^ {25} \) and Sastrasinh *et al.*\(^ {26} \) In general, binding characteristics in brush border membranes of the present study were comparable to those results, even though there were differences in methodology.

In brush border and basolateral membranes, treatment by phospholipase \( A_2 \) greatly increased gentamicin binding whereas no such binding by gentamicin was seen after treatment with proteolytic enzymes, trypsin and papain, and the sulphydryl reagent, \( p \)-chloromercuribenzoate. On the other hand, Sastrasinh *et al.*\(^ {26} \) reported that treatments with phospholipases A and C decreased gentamicin binding in brush border membranes. The reason for this discrepancy is unclear.

The binding of aminoglycosides to plasma membranes is thought to involve a charge interaction between polycationic drugs and anionic sites of the membranes. In this regard, we have estimated the relationship between gentamicin binding and membrane surface charge. Present data suggest that basolateral membranes contain more negative charge than brush border membranes and therefore the former interacted more effectively with gentamicin. Furthermore, the acidic phospholipid content of brush border membranes was increased by the incubation of brush border membranes with phosphatidylinositol and phosphatidic acid. The treatment of acidic phospholipids with brush border membranes increased the anionic surface charge and the gentamicin binding.

Present data showed that calcium decreased gentamicin binding to brush border membranes, probably by neutralizing the negative charge of the membranes. Recently Humes *et al.*\(^ {27} \) have reported that calcium is an effective inhibitor of gentamicin-renal membrane interaction and that oral calcium loading protects against the later stages of gentamicin nephrotoxicity.

As reported by Kaloyanides and colleagues,\(^ {6-7} \) it cannot be concluded that the accumulation of aminoglycosides in renal cortex was derived solely from absorption across brush border membranes of proximal tubular cells. Basolateral membrane transport may have contributed to the intracellular accumulation of aminoglycosides.\(^ {11,12} \) Our results indicate that the characteristics of gentamicin binding to both membranes were essentially similar. Therefore, these data may support the hypothesis that the renal ac-
cumulation of gentamicin reflects the transport of gentamicin across brush border and basolateral membranes of proximal tubular cells.

In conclusion, the results of the present study indicate that gentamicin binds to renal brush border and basolateral membranes in a similar fashion, and that this binding is due to a charge interaction between the polycationic drug and the anionic sites of the membranes. These findings may represent useful information for further investigation on the mechanism of renal accumulation of aminoglycosides and an approach to reducing nephrotoxicity.

REFERENCES


