BINDING OF AMINOCYCLITOL ANTIBIOTICS TO KIDNEY AND INTESTINAL BRUSH BORDER MEMBRANES

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Binding of aminocyclitol antibiotics to intestinal and kidney brush border membranes has been studied *in vitro* by means of vesicular preparations. The binding is rapid, reversible, specific, saturable and has a high affinity. To both tissues, gentamicin and sisomicin bind to a single binding site or receptor. These antibiotics demonstrate increased binding under conditions of increasing pH. Membrane binding disappears when the vesicle proteins are denatured with TCA. A significant reduction in aminocyclitol binding after treatment of vesicles with papain indicates that a portion of the binding receptor protein is exposed to the outer surface of the brush border membrane. The accumulated evidence suggests that the nature of the binding mechanism is not a simple electrostatic interaction between the antibiotic's charged amino groups and the polyanions of the membrane. Alternatively, a specific membrane structure is required for binding whose characteristics reflect a drug-receptor interaction. Receptor binding is characterized as being saturable, reversible, and specific; all of which have been demonstrated for aminocyclitols and brush border membranes.

Studies have been undertaken in the past to examine the nature of the binding of aminocyclitol antibiotics to various tissue systems, in efforts to understand the cytotoxicity of these compounds. While aminocyclitols are reported to have negligible binding to serum proteins,[1,2,3] they do bind to whole bacterial cells[4,5] and tissue homogenates.[6] Moreover, patients with normal renal function, after treatment with gentamicin, show measurable levels of this antibiotic in the urine for ten days or more after the final dose, suggesting that the drug can be tissue bound. Gentamicin binding to tissues is also suggested by the biphasic curve of the excretion of the drug[7,8]. In such experiments, the degree of antibiotic-tissue interaction has been assessed through correlation with the extent of antibacterial activity, as measured by microbiological assays.

Kidneys, and the renal cortex in particular, are the major site of the deposition of gentamicin, as shown in studies in humans[9] and experimental animals.[10] Renal deposition can account for 40% of the antibiotic present in the body. Specificity of gentamicin accumulation by rat renal cortex has also been demonstrated by KWO and HOOK.[11] Their data also suggest specific binding sites for aminoglycosides present on the surface of renal proximal tubules. In the present investigation, binding of aminocyclitol antibiotics to both kidney and intestinal vesicles *in vitro* has been studied quantitatively. Also, some aspects of the nature of aminocyclitol-membrane binding have been characterized.

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Methods

Materials

All chemicals used were of reagent grade quality, obtained from local suppliers: Fisher Scientific Co., Fair Lawn, N. J.; J. T. Baker Chemical Co., Phillipsburg, N. J.; Merck and Co., Rahway, N. J., U.S.A.

Special reagents and enzymes were obtained from Sigma Chemicals, St. Louis, Mo. and Calbiochem, San Diego, Ca., U.S.A. The following antibiotics were obtained in pure form of known potency: Gentamicin sulfate (Batch No. GMS-IM-13) and sisomicin sulfate (Batch No. S162–781) were supplied by the Schering Corp., Bloomfield, N. J., U.S.A. Streptomycin sulfate (Batch No. H9177) and kanamycin sulfate (Batch No. 550601) were supplied by China National Chemicals Import and Export Corp., Peking, China.

Radioactive [^{14}C]gentamicin and [^{14}C]sisomicin were provided by Prof. C. P. SCHAFFNER of Rutgers University, U.S.A. The compounds are specifically labelled in their ring methyl groups, and are produced by growing suitable cultures of *Micromonospora* in the presence of [^{14}C]methionine. The labelled gentamicin had a specific activity of 12.5 mCi/mmole; and the labelled sisomicin had a specific activity of 18.9 mCi/mmole. The labelled aminocyclitols were found to be chromatographically pure, using Sephadex G-10 columns. Monitoring the radioactivity eluted from the column using either sisomicin or gentamicin preparations revealed a single peak. On the basis of molecular weights, therefore, the radiolabelled materials are composed only of trisaccharide material without the presence of extraneous labelled disaccharide and monosaccharide material. The level of sensitivity was judged to be 0.003 ~ 0.006% on the basis of the ratio of cpm base line/cpm peak height.

The labelled compounds were also subject to analysis by thin-layer chromatography by the method of MAEHR and SCHAFFNER[12]. Additional scored TLC plates were spotted with ^{14}C gentamicin and ^{14}C sisomicin and scanned for radioactivity. Each scored column was divided into 1/2 cm fractions, each of which was individually scrapped and counted for radioactivity. The pattern of radioactivity corresponded to the three major gentamicin components (comprising 89% of the plate radioactivity), and to the two minor components of the gentamicin C complex (gentamicin C_{2a} and C_{2b}) at lower Rf values, and comprising 4.5% of the plate radioactivity. These results are consistent with the data reported by BYRNE[13] for preparation of the gentamicin C-complex-methyl-^{14}C.

Experimental

Preparation of Brush Border Vesicles. Both kidney and intestinal brush border membrane vesicles were prepared by the method of MALATHI *et al.*,[14] using white New Zealand male rabbits. The method is based on Ca precipitation of contaminating membranes. The animals had free access to food and water, and were sacrificed by a sharp blow to the back of the neck. Our mannitol - tris, and sorbose - tris buffers contained added tetracycline·HCl at 4 μM concentration, to reduce bacterial contamination. The purity of the membrane preparation was tested by assaying for the same brush border marker enzymes as described by MALATHI.[14]

Protein Estimation: Protein was estimated by the method of LOWRY *et al.*,[15] using crystalline bovine serum albumin as a standard.

Binding Assays: The experimental system involves studying the binding of [^{14}C] gentamicin and [^{14}C] sisomicin to vesicles prepared from kidney proximal tubules or intestinal mucosa. The vesicles (60 λ) are incubated in a large volume (1 ml) of cold isotonic solution containing $HgCl_2$, KI, and phlorizin for 30 minutes, for the purpose of inhibiting uptake. An aliquot (60 λ) of reaction mixture containing NaSCN, [^{14}C] gentamicin (or (^{14}C) sisomicin), and sorbitol-tris buffer at pH 7.0 is added to the vesicles. Immediately thereafter, the mixture is filtered through a Millipore filter. The filter is washed with three volumes of cold isotonic buffer, dried, and immersed in BRAY's solution (New England Nuclear, Boston, Mass.) and counted in a Beckman LS-150 Liquid scintillation counter.

Treatment of Vesicles with Papain

A stock solution of papain (Worthington, 850 U/ml) is diluted to 10 U/ml with 100 ml diluent solution of the following composition and prepared fresh: 1 mM EDTA, 60 μM mercapto-ethanol and 5 mM cysteine·HCl. The enzyme suspension is activated by incubating with diluent at room temperature

for 1/2 hour.

Each membrane treatment with papain was performed as follows: A solution of 100 λ of intact vesicles (kidney or intestine) was mixed with 100 λ of sorbose (50 mM)-tris (2 mM) buffer pH 6.0. To this was added 16 λ of the papain solution (10 activity units/ml). Separate 60 λ aliquots were dispensed into culture tubes and put on ice. To each tube, 1 ml stop solution was added, and incubated at 0°C for 1/2 hour, followed by a binding assay as previously described.

Preparation of Intestinal Brush Borders

Intestinal brush borders were prepared from a rabbit according to the method of MILLER and CRANE.[16]

Results

Extent of Binding

Binding studies were performed with kidney and intestinal vesicles for both gentamicin and siso-

Fig. 1. SCATCHARD plot of gentamicin binding to kidney vesicles.
Binding coefficient, Kd=69.57 nM^{-1}. X'=No. of binding receptors=32.10 nM/mg protein.

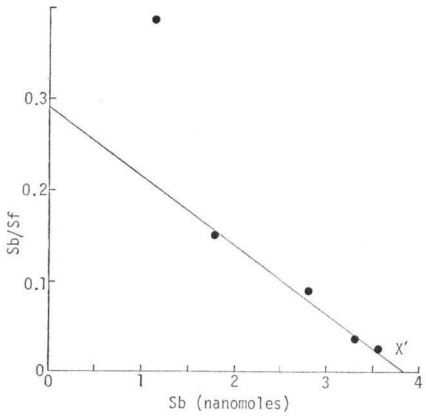


Fig. 2. SCATCHARD plot of gentamicin binding to intestinal vesicles.
Binding coefficient, Kd=215.24 nM^{-1}. X'=No. of binding receptors=50.32 nM/mg protein.

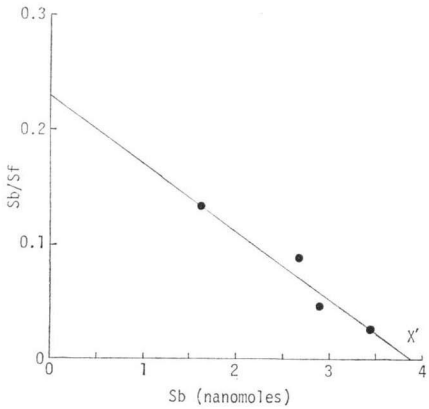


Fig. 3. SCATCHARD plot of sisomicin binding to kidney vesicles.
Binding coefficient, Kd=146.96 nM^{-1}. X'=No. of binding receptors=37.45 nM/mg protein.

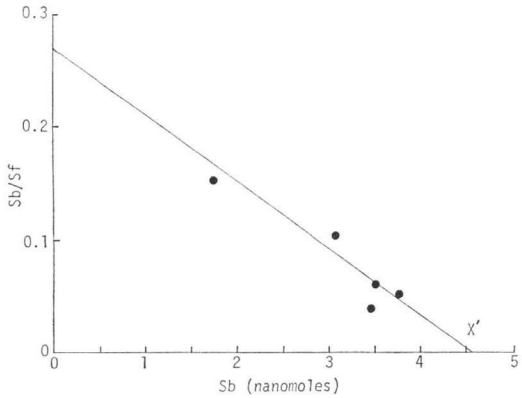
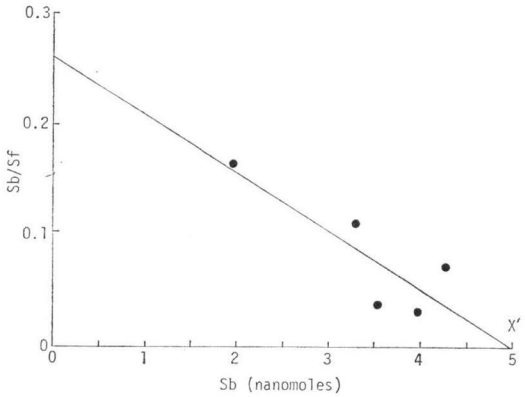


Fig. 4. SCATCHARD plot of sisomicin binding to intestinal vesicles.
Binding coefficient, Kd=248.43 nM^{-1}. X'=No. of binding receptors=64.52 nM/mg protein.



micin, as described in the experimental section. The amount of labelled antibiotic was increased, while the concentration of the brush border membrane protein was held constant. The data was analyzed by SCATCHARD plot analysis. The resultant four plots are depicted in Figs. 1~4. A plot of the ratio of bound to free antibiotic *versus* the concentration of bound antibiotic is linear in all four cases. The linear plots indicate that in both kidney and intestinal brush border membranes, both gentamicin and sisomicin bind to a single binding site or receptor.

The values for X' (*i.e.* the total concentration of binding receptors) in Figs. 1~4 show that there is a similar amount of binding in kidney vesicles for both gentamicin and sisomicin. While the concentration of binding receptors for both antibiotics in the kidney vesicles is on the same order of magnitude, there are about 16% more receptors for sisomicin over gentamicin.

There is a greater amount of binding per mg of protein for the aminocyclitol antibiotics in the intestine than the kidney, as the intestinal vesicles have X' values about 70% higher than those for kidney vesicles. However, this comparison rests on the assumption that the two vesicles contain the same amounts of protein. There is a similar amount of binding in intestinal vesicles for both gentamicin and sisomicin.

The *Kd* values show there is a similar degree of binding affinity in kidney vesicles for both gentamicin and sisomicin. The dissociation constants for both antibiotics are on the same order of magnitude, with gentamicin having a tighter degree of binding (*i.e.* lower *Kd* value). However, in the intestine, the two aminocyclitols have similar degrees of binding affinity. Intestinal binding affinity for sisomicin and gentamicin is lower than the binding affinity for both gentamicin and sisomicin in kidney vesicles.

Effects of pH and Ions Upon Binding

With kidney and intestinal vesicles, both gentamicin and sisomicin showed increased membrane binding with increasing pH. The corresponding results are shown in Figs. 5 and 6. There is maximum binding at pH values corresponding to the aminocyclitols having a less positive change. The binding curves imply a titration curve pattern, with the further suggestion that ionization inhibits a binding component which could be hydrophobic in nature.

Fig. 5. pH Dependence of gentamicin binding in intestinal vesicles.

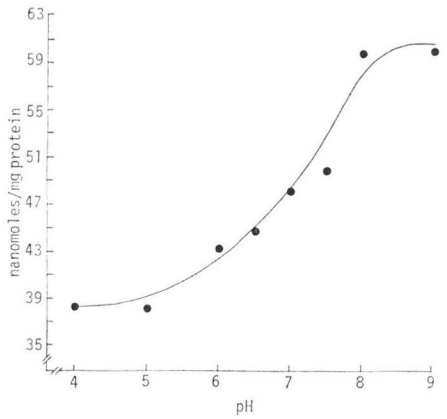
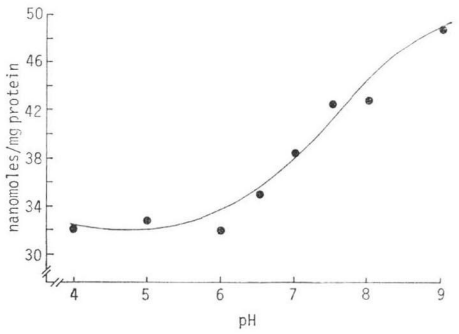


Fig. 6. pH Dependence of sisomicin binding in kidney vesicles.



Both gentamicin and sisomicin show reduced binding to vesicles under conditions of increasing ionic strength. The two compounds were each tested at a 1-mM concentration over a range of 100 μM to 10 mM ionic solutions (*i.e.* NaSCN). Moreover, the results are very similar, whether a solution of either NaSCN or KSCN of the same ionic strength is used. Additional experiments revealed that there was no significant reduction in gentamicin binding to kidney vesicles when washed with sucrose solutions of varying osmolarities (52 mosm to 1,000 mosm). Therefore, the described reduction in binding is not due to specific cation inhibition nor to osmolarity, but rather can be genuinely ascribed to ionic strength. These results are shown in Table 1.

Effect of Calcium Ions on Aminocyclitol Binding to Kidney

Gentamicin is known to form chelation complexes as free bases with divalent cations.[17~19] Since the preparation of vesicles involves a calcium chloride step to precipitate cellular organelles, the membranes retain a large calcium content. When the vesicles were washed with EGTA (ethylene glycol-bis-(β-aminoethylether)-N,N-tetraacetic acid), a calcium binding chelator, considerable reduction of binding is obtained. The results are shown in Table 2. At a concentration of 100 μM gentamicin, the amount of specific binding to membrane vesicles is reduced by over 66% after one washing with EGTA, from a level of 25.442 nm/mg protein down to 7.981 nm/mg protein. Further washings at the vesicles with EGTA reduced the amount of net binding by only smaller additional increments (*i.e.*, a further 8% reduction to 6.016 nm/mg protein). The data shows that Ca^{+2}

Table 1. Effect of ionic strength on binding of aminocyclitol antibiotics to kidney and intestinal vesicles at pH 7.0.

(A) Kidney vesicles

	Conc. of ^{14}C-aminocyclitol incubated with vesicles						
Binding level (nm/mg protein)	Control	Washed with 100 μM NaSCN	Washed with 1 mM NaSCN	Washed with 10 mM NaSCN	Washed with 100 μM KSCN	Washed with 1 mM KSCN	Washed with 10 mM KSCN
Gentamicin (1 mM)	33.3	28.899	22.228	5.343	29.753	20.165	4.896
Sisomicin (1 mM)	36.112	29.316	22.858	10.119	32.123	28.332	9.489

(B) Intestinal vesicles

	Conc. of ^{14}C-aminocyclitol incubated with vesicles						
Binding level (nm/mg protein)	Control	Washed with 100 μM NaSCN	Washed with 1 mM NaSCN	Washed with 10 mM NaSCN	Washed with 100 μM KSCN	Washed with 1 mM KSCN	Washed with 10 mM KSCN
Gentamicin (1 mM)	58.167	53.050	43.804	13.257	57.888	42.715	11.787
Sisomicin (1 mM)	36.86	36.688	31.511	12.209	40.115	41.073	12.531

Table 2. Effect of EGTA washing upon gentamicin binding to kidney vesicles.

Treatment	Net binding in nm/mg protein*
Vesicles not treated with EGTA	25.442
Vesicles washed with EGTA 1×	7.981
Vesicles washed with EGTA 2×	9.013
Vesicles washed with EGTA 3×	6.016

* Total activity minus the gentamicin retained on the Millipore without the vesicles.

Table 3. Effect upon membrane binding of washing filtered intestinal vesicles with unlabelled gentamicin.

Conc. of ^{14}C gentamicin incubated with vesicles	Binding after wash with 1 ml	Binding after wash with 2 ml	Binding after wash with 3 ml	Control binding (no gentamicin wash)
Gentamicin (100 μM)	3.418	3.270	1.592	25.958
Gentamicin (1 mM)	20.04	13.216	8.731	61.182

NB: All figures refer to binding activity measured in units of nm/mg protein.

is involved in aminocyclitol binding to vesicles. However, EGTA treatment enables us to measure a high level of aminocyclitol binding not dependent on the Ca^{+2} content of the vesicle preparation.

Differentiation of Aminocyclitol Binding from Transport

Two experiments were performed, one with intestinal brush borders and the other with kidney vesicles, to demonstrate that the activity retained on the filter represented membrane binding, and not uptake into vesicular space.

Unlike vesicles, intestinal brush border preparations are open and do not accumulate substrate. Therefore, any activity retained on the Millipore filter is likely to be bound. A glucose transport assay (100 μM) with a 100 mM NaSCN gradient was carried out with intestinal brush borders. When the net activity on the filter (*i.e.* glucose filtered without brush borders) is subtracted, there was no glucose binding, nor uptake over timed transport assay periods of up to 15 minutes. Correspondingly, when a gentamicin (100 μM) transport assay was carried out in the same system, there was significant membrane binding (4086 pm/mg protein) but no net uptake over timed incubation periods of up to two minutes. This result indicates that with intestinal brush borders preparations, binding can occur, and the filtered activity does not represent uptake into vesicular space.

When kidney vesicles are filtered (using Millipore filters) and then washed with water, the hypoosmotic conditions cause the vesicles to burst. In the absence of surface or internal membrane binding, any activity originally taken up into the vesicles would be expected to be washed from the filter upon disruption or hypoosmotic bursting of the vesicles. To demonstrate this behaviour, vesicles were allowed to take up glucose for 30 and 60 seconds. At 100 μM concentration, glucose uptake into kidney vesicles was 17 pm/mg protein after incubation for thirty seconds, and 8.5 pm/mg protein was taken up after incubation for sixty seconds. After hypoosmotic bursting, the amount of glucose transported into kidney vesicles is reduced to zero, for both incubation periods. Having verified the validity of this test system with D-glucose, similar experiments were carried out with gentamicin. When a standard binding assay was conducted with gentamicin (100 μM) and kidney vesicles, a binding value of 2,209 pm/mg protein was obtained. When the same binding assay was conducted under conditions of hypoosmotic bursting, the activity retained on the filter remained nearly constant (2,046 pm/mg protein). The conclusion is that the activity retained on the filtered vesicles is genuine membrane binding, and does not represent gentamicin transported into vesicles.

It was also observed that when gentamicin (100 μM) was incubated with kidney vesicles for periods of 30 and 60 seconds, and then subjected to conditions of hypoosmotic bursting, the amount of gentamicin now taken up into the vesicles (as measured by the activity retained on the filter) increased by 28% and 48% respectively. The implication is that the net increase of activity is due to internal binding

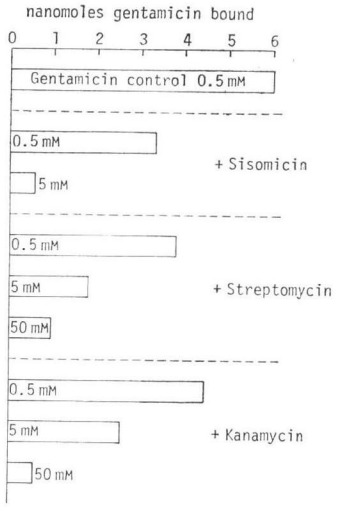
of the gentamicin to the disrupted membrane. Previous data had suggested that membrane binding became saturated at a level of about 300 μM gentamicin. Therefore, at higher antibiotic concentrations, a diffusion gradient would lead to additional accumulation of activity into vesicular space. Indeed, at higher gentamicin concentrations (500 μM and 1,000 μM), there was a significant net reduction in the amount of gentamicin bound and transported following hypoosmotic bursting. This reduction is ascribed to the disappearance of vesicular space, and the consequent elimination of antibiotic which had diffused into vesicular space or had become unbound.

Aminocyclitol Antibiotic—Membrane Binding is Reversible

^{14}C-Gentamicin was incubated with kidney vesicles in the presence of increasing concentrations of unlabelled glucosamine. No inhibition of gentamicin binding was observed, even with a 100: 1 ratio of glucosamine/^{14}C gentamicin.

In another experiment, separate aliquots of ^{14}C gentamicin were incubated with kidney vesicles in the presence of increasing concentrations of unlabelled sisomicin, kanamycin, and streptomycin. The results are shown in Fig. 7. All three amino cyclitol antibiotics caused inhibition of gentamicin binding. The degree of inhibition increased with increasing concentration of the inhibitor. At equimolar concentrations, sisomicin showed the greatest inhibition of gentamicin binding (45%), followed by streptomycin (37%) and kanamycin (26%). The same relative relationship of inhibitor potency was observed when these aminocyclitol antagonists were incubated with gentamicin in a molar ratio of 10: 1. *Ki* values were determined for each of the aminocyclitol inhibitors, and found to be: sisomicin 276 nm; kanamycin 325 nm; streptomycin 461 nm.

Fig. 7. Inhibitory effect of aminocyclitol antibiotics upon gentamicin binding in kidney vesicles.



Effect of Papain on Aminocyclitol-Membrane Binding

The results of these experiments are shown in Table 4. In both intestinal and kidney vesicles, the level of aminocyclitol binding is over ten fold greater than found for glucose, and about forty times greater than the binding levels found for glucosamine. Upon treatment with papain, at varying incubation periods for up to ten minutes, binding levels for both glucose and glucosamine are essentially unchanged. In contrast, treatment with papain causes a large inhibition of aminocyclitol binding in both kidney and intestinal vesicles.

In both membrane tissues, the effect of papain inhibition is rapid, the reaction being essentially complete after two minutes. In kidney vesicles, gentamicin binding is inhibited by about 34%, while sisomicin binding undergoes a smaller degree of inhibition at about 25%. Compared to kidney membrane binding, papain treatment has a greater inhibitory effect upon aminocyclitol binding to intestinal membranes. With intestinal vesicles, gentamicin binding and sisomicin binding show similar inhibition levels after papain treatment, to the extent of 44~48%.

Table 4. Effect of papain on aminocyclitol membrane binding to kidney and intestinal brush border vesicles.

	Substrate	Treatment	Binding nm/mg protein	% inhibition of binding
(I) Intestinal vesicles	Glucose (100 μM)	Control	3.926	—
		Papain 2 min.	4.462	—
		Papain 5 min.	4.520	—
		Papain 10 min.	4.332	—
	Glucosamine (100 μM)	Control	0.811	—
		Papain 2 min.	0.420	48.2 %
		Papain 5 min.	0.594	26.1 %
		Papain 10 min.	0.941	—
	Gentamicin (100 μM)	Control	30.023	—
		Papain 2 min.	19.532	35.1 %
		Papain 5 min.	19.764	34.2 %
		Papain 10 min.	19.865	33.9 %
	Sisomicin (100 μM)	Control	31.486	—
		Papain 2 min.	24.299	22.9 %
		Papain 5 min.	23.169	26.5 %
		Papain 10 min.	23.517	25.4 %
(II) Kidney vesicles	Glucose (100 μM)	Control	1.722	—
		Papain 2 min.	1.819	—
		Papain 5 min.	1.770	—
		Papain 10 min.	1.805	—
	Glucosamine (100 μM)	Control	0.340	—
		Papain 2 min.	0.305	10.3 %
		Papain 5 min.	0.361	—
		Papain 10 min.	0.326	4.6 %
	Gentamicin (100 μM)	Control	12.221	—
		Papain 2 min.	6.312	48.4 %
		Papain 5 min.	6.513	46.8 %
		Papain 10 min.	6.117	50 %
	Sisomicin (100 μM)	Control	12.526	—
		Papain 2 min.	7.221	42.4 %
		Papain 5 min.	6.860	45.3 %
		Papain 10 min.	7.103	43.3 %

Discussion

When the interaction of aminocyclitol antibiotics was tested with isolated kidney vesicle preparations, the first observation made was the high degree of antibiotic binding (28 nm/mg protein). The binding in both kidney and intestinal membrane vesicles is rapid; it is saturable; and is not inhibited by agents such as KI, mercuric ion, and phlorizin.

The extent of aminocyclitol membrane equilibrium binding was analyzed by SCATCHARD plots. The linear plots obtained in all four cases show that in both kidney and intestinal brush border membranes, both gentamicin and sisomicin bind to a single binding site or receptor. Moreover, the concen-

tration of aminocyclitol binding receptors is high (*i.e.*: 50 nm/mg protein) and the antibiotic binding receptor has a high affinity (100 nM^{-1}), and of a magnitude comparable to those reported for hormonal receptors (*i.e.*: $Kd=2$ nM^{-1} for cardiac β-adrenergic receptors[20,21]). In contrast to our data, KORNGUTH reports[22] an association constant at 1.5×10^{4} M^{-1} for gentamicin binding to kidney mitochondrial fractions. However, his binding assay was carried out by incubating gentamicin with cell fractions at 37°C for 30 minutes, possibly causing the measurement of diffusion and transport into the organelle in addition to binding. It would appear that for each antibiotic (gentamicin and sisomicin), there is close agreement in the number or concentration of binding receptors for each tissue. However, the large differences between the binding affinities for each tissue suggests that the binding receptors are different in the kidney *vs.* the intestine.

JUST *et al.*[23] have previously reported gentamicin binding in kidney brush border membranes, using ^{3}H gentamicin. They reported renal gentamicin binding as being of low affinity and high capacity. In SCATCHARD plot binding data, a straight line was obtained with a binding capacity (X') of 18.2 nm, and an affinity constant (Kd) of 43 mM^{-1}. In contrast, we have reported a higher binding capacity (32.1 nm) and a higher binding affinity (120.28 nM^{-1}). We suggest that these differences may arise from several factors.

In this regard, the equilibrium binding measurements were performed differently by the two groups. We assayed binding at a very short incubation period, using the technique of Millipore filtration to trap labelled gentamicin bound to the filtered vesicles. In contrast, JUST incubated brush borders plus unlabelled gentamicin for a long (30 minutes) period, followed by a second 30 minute-incubation period with labelled gentamicin. Our work has also revealed that in addition to binding, gentamicin is also transported into kidney brush border membranes.[24,25] These two processes would not be differentiated with JUST's long incubation periods. Moreover, JUST's preparation of kidney brush borders is also likely to contain some vesicles. Therefore, his measured binding activity would also include gentamicin transported by diffusion into the vesicles. We suggest that the lower affinity recorded by JUST and also by KORNGUTH[22], could be ascribed to measurement of gentamicin transport and not binding, at least in part.

Because aminocyclitol antibiotics are charged basic molecules at physiological pH, several groups have suggested that their binding to membranes is primarily electrostatic in nature. The polycationic nature of aminocyclitols has also been suggested to contribute to their tissue affinity and toxicity through binding to biological polyanions; such as the acidic lipoproteins in the lysosomes of kidney proximal tubules.[2] JUST[23] has suggested a correlation between the binding of gentamicin to renal brush border membranes, and the compound's basicity; by noting that the degree of aminocyclitol binding increases with an increasing number of amino groups in the molecule. He also suggests that gentamicin's polycationic nature is an important component of renal brush border membrane binding and pinocytosis. ALEXANDER,[26] DEGUCHI,[27] and AKIYAMA[28] by studying model *in vitro* physico-chemical systems, have also proposed that aminocyclitols bind largely by electrostatic interactions to the membrane. They have proposed that tissue affinity and toxicity of these antibiotics is due to their polycationic character, through binding to biological polyanions such as acid mucopolysaccharides.

However, our observations that the amino-cyclitols show increased membrane binding with increasing pH, make the postulate unlikely that aminocyclitol-membrane binding is primarily electrostatic in nature. That is, the low Km of binding, plus the observation that membrane binding is reduced to negligible levels when the vesicle proteins are denatured by TCA, lends support to alternative possibilities that binding may depend upon a receptor protein sensitive to pH, or may have important hydrophobic components. However, we feel that there may be an ionic factor involved in aminocyclitol binding to membranes as well. This is felt because the level of antibiotic binding can be reduced by washing the filtered vesicles with buffers of increasing ionic strength. Additional experiments revealed that this effect is not due to the osmolarity of the same buffers.

The observations that gentamicin (100 μM) binding to kidney vesicles was reduced by over 66% after one washing with EGTA (a calcium binding chelator), and that further washings of the vesicles with EGTA reduced the amount of net binding by only smaller additional increments, provide some support to the view that while Ca^{+2} is involved in aminocyclitol binding, there is also a high level of specific

binding of these antibiotics to brush border membranes which is not dependent on the Ca^{+2} content in the vesicles.

Other experiments showing that the level of ^{14}C gentamicin binding in intestinal vesicles is reduced upon washing the filtered vesicles with increasing concentrations of unlabelled gentamicin, demonstrates that aminocyclitol binding to brush border membranes is reversible.

In addition to being reversible, aminocyclitol binding to membranes is specific. The absence of binding inhibition by a monosaccharide amino sugar (*i.e.* glucosamine) has been also observed by KORNGUTH *et al.*,[22] and suggests that the amino groups are not a primary component of the receptor drug interaction. However, other aminocyclitol analogs of related structures exhibit competitive binding antagonism to gentamicin, which suggest that the stereo chemical structure of the aminocyclitol is essential for binding. If the basic amino groups were a primary factor in binding specificity, then kanamycin (*i.e.* four amino groups) would be expected to have a greater inhibitory effect on gentamicin binding than streptomycin (*i.e.* two guanidino groups). However, Fig. 7 shows that the opposite result was found, further supporting the view that the gentamicin-membrane receptor binding is primarily dependent on the antibiotic's stereochemical structure; and not on the basicity of the compound's amino groups.

KUNIN[6] had measured the inhibition of aminocyclitol antibiotic activity in the presence of kidney homogenate, and found that binding descended with decreasing toxicity of aminocyclitols: gentamicin> kanamycin>streptomycin. He correlated this order with the decreasing number of amino groups. However, studies with whole tissue homogenates may reflect properties of a variety of membranes, and may not distinguish membrane binding from transport; nor provide clear information regarding the nature of aminocyclitol-receptor binding to the brush border membrane. Binding to other renal membranes, or membrane transport may have a more direct bearing upon aminocyclitol nephrotoxicity.

The observation that treatment of intact kidney and intestinal vesicles with papain caused a significant reduction of aminocyclitol binding (Table 4), indicates that the papain sensitive portion of the binding receptor protein is exposed to the outer surface of the brush border membrane. The loss of binding activity resulting from protease treatment is taken to mean that papain destroys the high affinity gentamicin and sisomicin binding receptors.

However, not all the antibiotic binding is abolished after papain treatment. At least three possibilities can be suggested to explain this phenomenon:

(1) Incomplete digestion: The conditions of the experiment are not sufficient to complete degradation of the aminocyclitol binding protein(s), resulting in only partial inactivation of the available receptors. This was considered a possibility, as the degree of papain induced binding inhibition was reduced by up to 30% when the aminocyclitol substrate concentration was increased from 100 μM to 1 mM. The recorded level of binding inhibition was complete after two minutes of papain incubation, and did not increase with longer incubation periods.

(2) Membrane damage: An alternative explanation is that the papain treatment has damaged the membrane and therefore altered its binding affinity for the antibiotic.

(3) Additional binding sites uncovered: Another possible explanation as to why papain treatment does not abolish all antibiotic-membrane binding is that proteolytic treatment uncovers additional and previously unavailable binding sites on the membrane surface. It is also possible that the residual binding left after papain treatment may represent nonprotein binding of gentamicin to membrane phospholipids.

The accumulated evidence suggests that the nature of the binding interaction is not a simple electrostatic interaction between the antibiotics's charged amino groups and the polyanions of the membrane. Rather, a specific membrane structure is required for binding whose characteristics reflect a drug-receptor interaction. Receptor binding is characterized as being saturable, reversible, and specific; all of which have been demonstrated for aminocyclitols and brush border membranes.

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