

# Renal Ultrastructural and Biochemical Injuries Induced by Aminoglycosides

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Aminoglycoside-induced proteinuria may result from general renal damage or may reflect alterations in specific steps in the renal handling of proteins. To differentiate between the two possibilities, experiments were designed to quantify the effects of nephrotoxic doses of gentamicin, tobramycin and netilmicin in the intact rat, isolated perfused rat kidney (IPK) and kidney slices using the cationic low molecular weight protein lysozyme as a model protein. Each aminoglycoside was administered IP to male Wistar rats (15 or 30 mg/kg/day) for 5 or 7 days.

Scanning and transmission electron microscopy indicated that gentamicin and tobramycin induced a decrease in the number and diameter of endothelial fenestrae and degranulation of the myoepithelioid cells of the juxtaglomerular apparatus. Concurrently, gentamicin and tobramycin decreased the glomerular sieving coefficient of lysozyme from 0.8 to 0.6 and 0.5, respectively. Netilmicin did not affect the percentage reabsorption of lysozyme whereas gentamicin and tobramycin induced a 50% decrease in lysozyme reabsorption by the IPK. Gentamicin and tobramycin decreased equally lysozyme degradation by the IPK; this decrease was time- and dose-dependent when evaluated in slices from renal cortex. Perfusion of rat kidneys with gentamicin induced a dose-dependent decrease in reabsorption and catabolism of lysozyme.

In conclusion, these studies demonstrate that polycationic aminoglycosides alter ultrastructure and glomerular permeability, tubular reabsorption and intracellular digestion of proteins.

Aminoglycoside antibiotics are valuable therapeutic agents which are employed frequently in clinical management of gram-negative bacterial infections. Nephrotoxicity, a major side effect of aminoglycoside treatment, is usually characterized by a fall in glomerular filtration rate (GFR), decreased urinary concentrating capacity, proteinuria, enzymuria and ultrastructural alteration of proximal tubular cells. For several years, efforts of various research groups have focused on detection and assessment of the mechanisms of aminoglycoside nephrotoxicity. Luminal uptake is the major route of aminoglycoside tubular reabsorption and subsequent intracellular accumulation (1-3). Contraluminal uptake of aminoglycosides (4,5) also appears to contribute to the accumulation of aminoglycosides. Although aminoglycosides inhibit Na, K-ATPase activity, it is unlikely that this is the primary mechanism of aminoglycoside nephrotoxicity (6). The effects of aminoglycosides on mitochondrial, (7) and lysosomal function (8-11) appear to play the

main role in aminoglycoside-induced nephrotoxicity. There is new evidence to support glomerular alterations induced by aminoglycosides (8), though this is still disputed (12-14). Low molecular weight proteins (LMWP) are well filtered by the glomerulus and almost totally reabsorbed by the tubule in control conditions. Renal handling of LMWP such as lysozyme and  $\beta_2$ -microglobulin appears to be an early target in aminoglycoside nephrotoxicity (15,16). A summary of some of our investigations on the effects of aminoglycosides on renal filtration, reabsorption and catabolism of proteins are presented herein.

## Glomerular Alterations

Decreased GFR is a common finding in patients and experimental animals treated with aminoglycosides. Treatment of male rats with a toxic dose (30 mg/kg/day) of aminoglycosides for 7, 14 and 21 days (Fig. 1) demonstrated that only gentamicin and netilmicin decreased GFR over the entire treatment period. The same *in vivo* experiments indicate that tobramycin failed to decrease GFR. After 14 days of treatment GFR showed a tendency to return to control in rats treated with netilmicin. No recovery of GFR was observed in gentamicin-treated rats. Gentamicin treatment of Munich-Wistar rats for 10 days at a similar

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dose (40 mg/kg/day) also induced a significant decrease in glomerular capillary ultrafiltration coefficient ( $K_f$ ) (12).

Since aminoglycoside treatment appeared to decrease tubular reabsorption of the low molecular weight protein lysozyme, investigation of the effects of aminoglycosides on glomerular filtration of this protein was necessary to

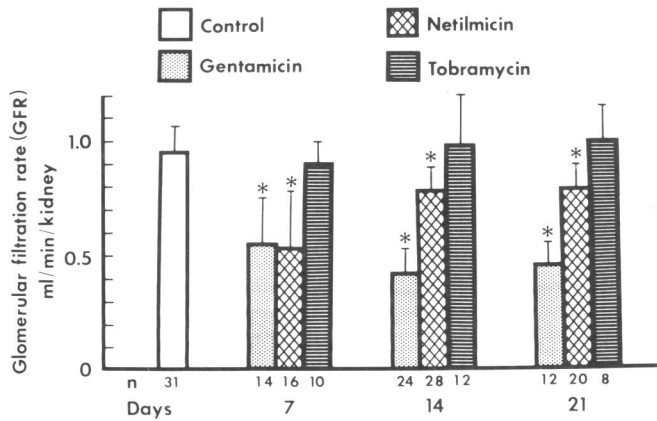


FIGURE 1. Effects of aminoglycosides on GFR in intact rats. Days = duration of aminoglycoside treatment,  $n$  = number of clearance periods. Significantly different from control ( $p \leq 0.05$ ). Bars represent mean  $\pm$  1 SD. From Cojocel and Hook (15). Reprinted with permission of Elsevier Scientific Publishers, Ireland, Ltd.

elucidate the mechanism by which aminoglycosides alter its renal reabsorption and catabolism. Thus, experiments with the isolated perfused kidney were designed to determine the glomerular sieving coefficient of lysozyme. At complete inhibition of tubular reabsorption of lysozyme the ratio of lysozyme clearance/inulin clearance ( $C_{LY}/GFR$ ) expresses the glomerular sieving coefficient of lysozyme ( $GSC_{LY}$ ). Following aminoglycoside treatment, sodium iodoacetate was added to the perfusate (3–5 mM) to completely inhibit tubular reabsorption of lysozyme (17). Aminoglycoside treatment decreased the  $GSC_{LY}$  from a control value of 0.8 to 0.6 and 0.5 in kidneys from

Table 1. Effect of aminoglycosides on the density of endothelial fenestrae (EF).

Treatment	EF density (25 cm <sup>2</sup> ) <sup>a</sup>	% of Control
Saline (Control)	99 $\pm$ 2 (n = 4)	100
Netilmicin	103 $\pm$ 2 (n = 4)	100
Gentamicin	82 $\pm$ 3* (n = 5)	82*
Tobramycin	77 $\pm$ 3* (n = 5)	77*

<sup>a</sup>The density of endothelial fenestrae was determined by counting the number of fenestrae on a 25 cm<sup>2</sup> area of prints from scanning electron microscopy. Each aminoglycoside was administered at a dose of 30 mg/kg/day for 7 days. Data are given as means  $\bar{X} \pm$  SD. Numbers in parentheses represent the number of rats.

\*Significantly different from control value ( $p < 0.05$ ).

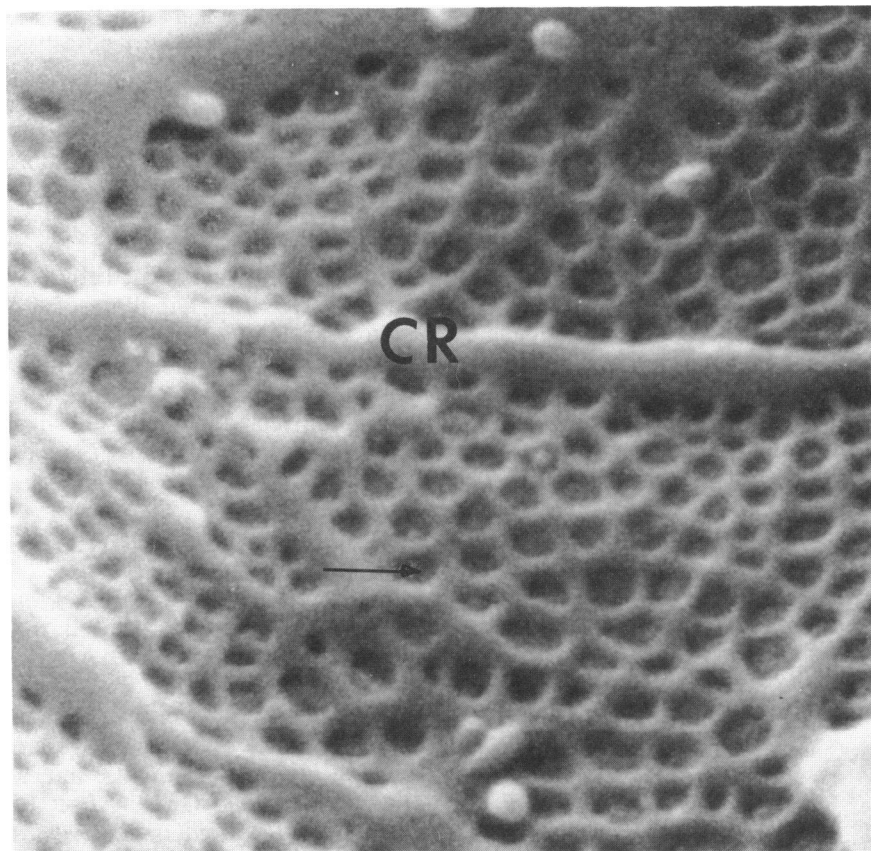
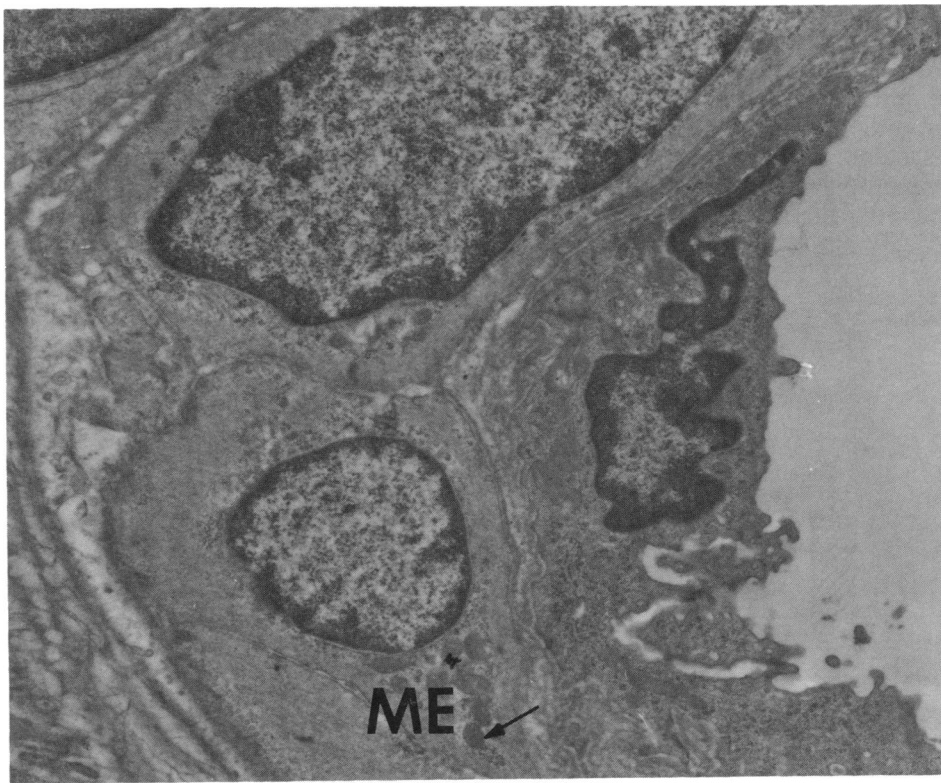


FIGURE 2. Glomerular ultrastructure by scanning electron microscopy. Scanning electron micrograph showing endothelial fenestrae (arrow) in control rats. Cytoplasmic ridges (CR) are noticeable.  $\times 28,000$ . From Cojocel et al. (8). Reprinted with permission of Academic Press.



(a)



(b)

FIGURE 3. Effect of gentamicin on glomerular ultrastructure. Transmission electron micrograph of the afferent arteriole of juxtamedullary apparatus from (a) control and (b) gentamicin-treated rats for 7 days (30 mg/kg/day). Myoepithelioid cells (ME) contain numerous electron dense cytoplasmic granules (arrows) in control rats while these granules are almost completely depleted in gentamicin-treated rats.  $\times 5470$ .

gentamicin-and tobramycin-treated rats, respectively; netilmicin had no effect. Scanning and transmission electron microscopy indicated that gentamicin and tobramycin altered the ultrastructure of the glomerular endothelium by reducing diameter and frequency of endothelial fenestrae (Fig. 2 and Table 1). Quantitative morphometric studies using data from scanning electron microscopy (Table 1) supported previous studies (18) and demonstrate that aminoglycoside treatment decreased the number of glomerular capillary endothelial fenestratae.

Glomerular functional and ultrastructural alterations caused by aminoglycoside treatment could be induced through a tubuloglomerular feedback mechanism mediated by the renin-angiotensin system which has been suggested to be related to the pathogenesis of acute renal failure by Thurau et al. (19). Consistent with this hypothesis, high doses of gentamicin (120 mg/kg/day) produced a significant increase in plasma renin activity and a concurrent decrease in GFR in rats (20). Lower doses of gentamicin or tobramycin (30 mg/kg/day) induced an almost complete depletion of cytoplasmic granules from the myoepithelial cells in the afferent arterial wall of the juxtamedullary apparatus as revealed by transmission electron microscopy (Fig. 3). At the same dose, depletion of cytoplasmic granules was less severe in netilmicin-treated rats. However, in these studies, there was no disintegration of the granules, elevated exocytotoxic excretion processes, or activation of rough endoplasmic reticulum. Thus, it is uncertain whether the depletion of the cytoplasmic granules from the myoepithelial cells after aminoglycoside treatment was related to activation of the renin-angiotensin system. The precise role played by this system in aminoglycoside-induced renal failure remains to be clarified by further studies.

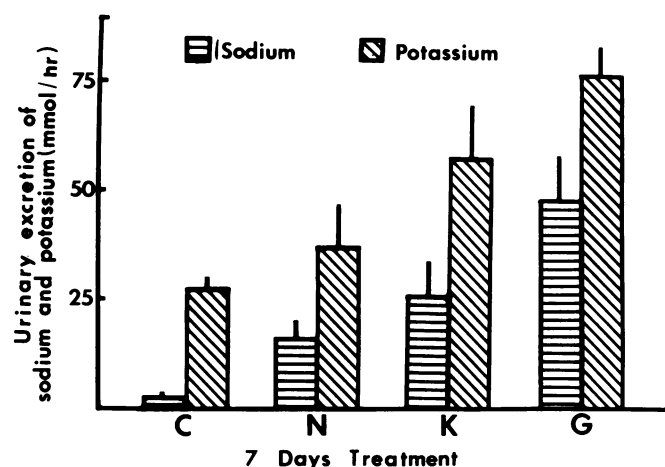


FIGURE 4. Effects of aminoglycosides on electrolyte excretion in intact rats. Excretion of sodium and potassium in control and aminoglycoside-treated rats for 7 days (30 mg/kg/day). C = control, G = gentamicin, K = kanamycin, N = netilmicin. From Cojocel and Hook (33). Reprinted with permission of Elsevier Science Publishers, B. V., Amsterdam.

## Impairment of Tubular Reabsorption

Treatment of patients or experimental animals with aminoglycoside antibiotics results in tubular cell injury and necrosis accompanied by enhanced excretion of glucose (21) or of LMWP such as  $\beta_2$ -microglobulin (16). These functional alterations suggest an impairment of tubular reabsorptive capacity. Indeed, increased urinary excretion of sodium and potassium are found in aminoglycoside-treated rats (Fig. 4). In these experiments gentamicin appeared to have a much more pronounced effect than netilmicin. The specific mechanism by which aminoglycosides decrease tubular reabsorption of electrolytes is still unknown.

Aminoglycoside treatment leads to an increased excretion of brush border enzymes (22), lysosomal enzymes (23) and LMWP such as  $\beta_2$ -microglobulin and lysozyme (16). Aminoglycoside-induced functional alterations were detected by measuring the urinary excretion of the LMWP lysozyme, the lysosomal enzyme *N*-acetyl- $\beta$ -D-glucosaminidase and total proteins in intact rats (Table 2). Gentamicin was more potent than netilmicin in decreasing tubular reabsorption of lysozyme and induced damage to cellular membranes causing leakage of the lysosomal marker enzyme NAG and an increase in total protein excretion (Table 2). Tubular reabsorption of lysozyme had a tendency to return toward control despite continued administration of gentamicin or tobramycin (15). Determination of the glomerular sieving coefficient of lysozyme after aminoglycoside treatment allowed a more accurate quantitation of tubular reabsorption of lysozyme (Table 3). Gentamicin and tobramycin (30 mg/kg/day) decreased endocytic reabsorption of lysozyme in isolated perfused kidneys to about 50% after 7 days of treatment. Since aminoglycosides and lysozyme are taken up into tubular

Table 2. Daily urinary excretion of endogenous lysozyme (LY), *N*-acetyl- $\beta$ -D-glucosaminidase (NAG) and total protein (P) in aminoglycoside-treated rats.

	Control	Netilmicin	Gentamicin
Ly, $\mu$ g/day	4.72 $\pm$ 0.50	5.95 $\pm$ 1.42	31.79 $\pm$ 18.99*
NAG, $\mu$ mole/hr/day	0.79 $\pm$ 0.08	0.78 $\pm$ 0.26	3.32 $\pm$ 1.76*
P, mg/day	6.22 $\pm$ 1.10	6.39 $\pm$ 0.38	38.3 $\pm$ 9.64*

\*All experimental data are given as mean value  $\pm$  SD. Each experimental group consisted of five rats. Each aminoglycoside was administered at a dose of 30 mg/kg/day for 7 days.

\*Data are significantly different from control ( $p < 0.05$ ).

Table 3. Effect of aminoglycosides on renal reabsorption of lysozyme by the isolated perfused rat kidney.

	Control	Netilmicin	Gentamicin	Tobramycin
$\bar{X} \pm$ SD	71.7 $\pm$ 3.0	69.7 $\pm$ 3.5	35.4 $\pm$ 6.8*	32.4 $\pm$ 10.5
<i>n</i>	5	6	5	5

\*Each aminoglycoside was administered at a dose of 30 mg/kg/day for 7 days. Lysozyme reabsorption is expressed as % of filtered load. *n* = number of kidneys.

\*Significantly different from control ( $p < 0.05$ ).

cells by endocytosis, it is very likely that the differential effects of aminoglycosides on tubular reabsorption of lysozyme represent specific alterations in proximal tubular function such as impairment of the endocytic protein reabsorption.

## Decrease of Lysosomal Proteolysis

Protein degradation in the kidney occurs mainly in lysosomes, organelles that are known to accumulate nephrotoxicants such as aminoglycosides. Gentamicin labilizes the lysosomal membrane causing increased release of lysosomal marker enzymes to the incubation medium *in vitro* (24) and increased excretion of lysosomal enzymes *in vivo* (25). Likewise, gentamicin treatment of rats led to a decrease of lysosomal protein degradation by renal cortical slices in a dose-related fashion (26) (Fig. 5). In these studies, the effects of gentamicin on lysosomal degradation of lysozyme by renal cortical slices was measured as free (nonprecipitable) radioactivity and was expressed as percentage of total radioactivity released to the incubation medium. The effects of gentamicin, tobramycin and netilmicin on renal protein degradation were compared (Fig. 6) in the isolated perfused rat kidney (8). In these studies

lysozyme degradation was estimated by the release of tyrosine into the perfusate during a 150-min perfusion period. Tyrosine concentration in the perfusate was quantified by high-pressure liquid chromatography (HPLC). The more nephrotoxic aminoglycosides, gentamicin and tobramycin, significantly decreased the release of tyrosine to the perfusate from a control value of 12.0 to approximately 4.5  $\mu\text{mole}$ . Netilmicin appeared to have no effect on renal degradation of lysozyme (Fig. 6). This study also showed that gentamicin and tobramycin had similar effects on renal protein catabolism. The decrease in renal catabolism of lysozyme may be due indirectly to decreases in glomerular filtration rate, the glomerular sieving coefficient of lysozyme, and impairment of tubular endocytic uptake of lysozyme. The direct effect of aminoglycosides on lysosomal protein degradation may be due to an increase in intralysosomal pH and subsequent decrease in the activity of proteolytic enzymes. Treatment of rats with gentamicin decreased the lysosomal activity of cathepsin B (11), a proteolytic enzyme responsible for the lysosomal breakdown of egg white lysozyme (27).

## Acute Effects of Gentamicin

Cationic LMWP such as lysozyme and cytochrome c compete for anionic binding sites at the proximal tubule brush border, the first step in endocytic tubular reabsorption (1,28,29). Similar competition between polycationic aminoglycosides appears to take place in

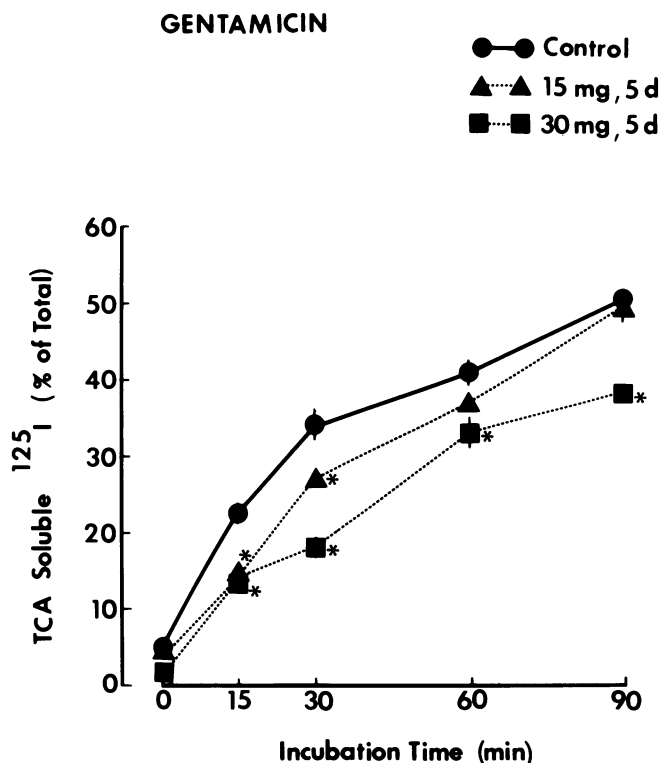


FIGURE 5. Dose-dependent inhibition of protein degradation by gentamicin. Protein degradation was measured as the release of trichloroacetic acid (TCA) soluble radioactivity from the slices into the medium and expressed as percentage of the total radioactivity. Bars represent mean  $\pm$  SEM of values obtained from 4 rats. Asterisk (\*) denotes significantly different from control ( $p < 0.05$ ). From Cojocel et al. (26). Reprinted with permission of the Society of Toxicology.

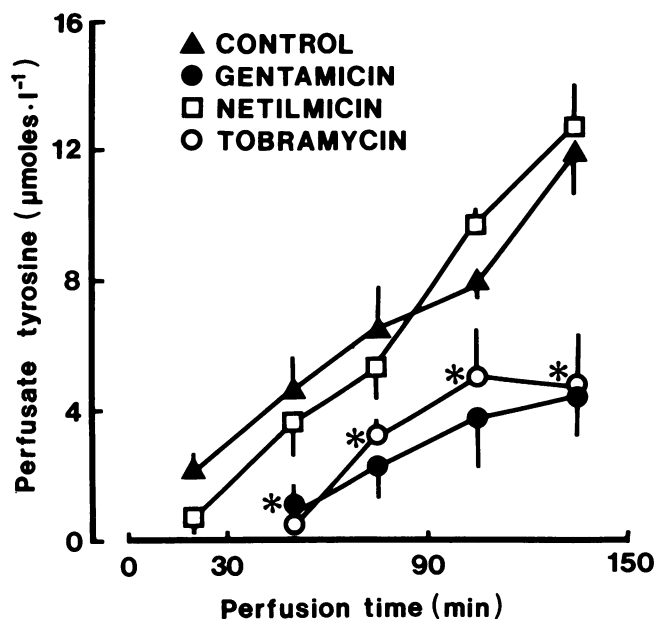


FIGURE 6. Effect of aminoglycosides on protein degradation. Renal degradation of lysozyme in control and aminoglycoside-treated rats (30 mg/kg/day) for 7 days. Values are represented as mean  $\pm$  1 SD of 5 or more kidneys. Asterisk (\*) denotes significantly different from control ( $p < 0.05$ ). From Cojocel et al. (8). Reprinted with permission of Academic Press.

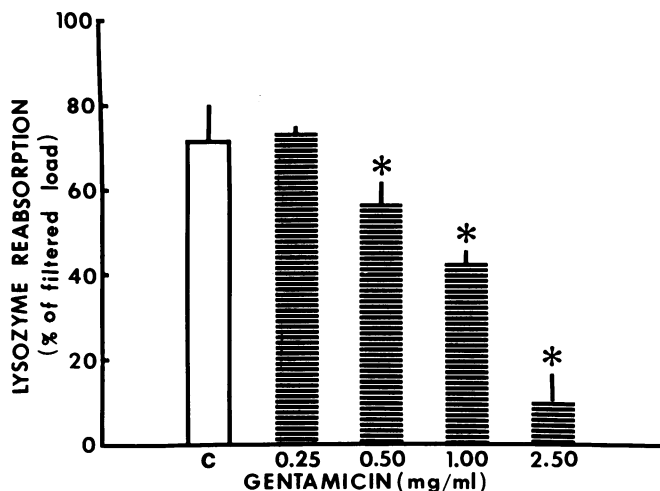


FIGURE 7. Effect of acute exposure to gentamicin on the reabsorption of lysozyme by the isolated perfused rat kidneys. C = control kidneys. Bars represent mean  $\pm$  SEM of 4 kidneys. Asterisk (\*) denotes significantly different from control ( $p < 0.05$ ). From Cojocel and Hook (32). Reprinted with permission of Elsevier Scientific Publishers, Ireland, Ltd.

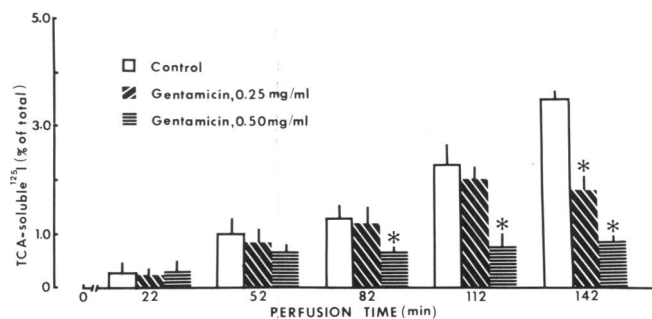


FIGURE 8. Catabolism of  $^{125}\text{I}$ -lysozyme by isolated perfused rat kidneys after acute exposure to gentamicin. Catabolism of  $^{125}\text{I}$ -lysozyme was measured as release of nonprecipitable  $^{125}\text{I}$ -radioactivity by TCA (trichloroacetic acid) to the perfusate. Bars represent mean  $\pm$  SEM of 4 kidneys. Asterisk (\*) denotes significantly different from control ( $p < 0.05$ ). From Cojocel and Hook (32). Reprinted with permission of Elsevier Scientific Publishers, Ireland, Ltd.

the processes of binding to the brush border (2) and subsequent renal accumulation (30). Since renal absorption of lysozyme takes place via endocytosis (31), the pharmacological interaction between lysozyme and gentamicin molecules in the early time after gentamicin infusion is very likely to affect tubular reabsorption and intracellular catabolism of lysozyme in the absence of tubular damage.

Isolated rat kidneys were perfused with gentamicin at concentrations of 0.25, 0.50, 1.0 and 2.5 mg/mL. A dose-dependent decrease in percentage reabsorption of lysozyme (Fig. 7) showed that gentamicin inhibits tubular reabsorption of lysozyme in a dose-dependent manner (32). Renal catabolism of  $^{125}\text{I}$ -lysozyme to smaller degradation products was measured as non-

precipitable radioactivity ( $^{125}\text{I}$  and  $^{125}\text{I}$ -monoiodotyrosine) released to the perfusate. Perfusion of the kidneys at a perfusate concentration of gentamicin of 0.5 mg/mL and higher induced almost complete inhibition of renal degradation of lysozyme (Fig. 8). This inhibition could be due primarily to inhibition of tubular reabsorption of lysozyme by gentamicin (Fig. 7) and additionally to the inhibition of enzymatic activity of lysosomal proteases responsible for the lysosomal catabolism of lysozyme.

In summary, aminoglycosides act as nephrotoxicants at the glomerulus, by reducing the glomerular filtration of inulin and cationic proteins, at the tubule by inhibiting the endocytic reabsorption, and at the lysosome, by decreasing the proteolytic activity of lysosomal enzymes.

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