# Inhibition of Apical Membrane Enzyme Activities and Protein Synthesis by Gentamicin in a Kidney Epithelial Cell Line LLC-$PK_1$

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**The mechanism of a gentamicin-induced decrease in apical membrane enzyme activities was investigated in LLC-$PK_1$ cells. Increasing activities of apical membrane enzymes (alkaline phosphatase, aminopeptidase, and $\gamma$-glutamyltransferase) were markedly suppressed by gentamicin during growth in culture. On the other hand, a lesser effect was observed when the activities of these enzymes were decreasing or relatively constant. Gentamicin treatment decreased the maximal enzyme activities of alkaline phosphatase and aminopeptidase, indicating that the number of active enzyme molecules in the apical membrane was decreased by gentamicin. [$^3$H]Leucine incorporation in LLC-$PK_1$ cells was inhibited by gentamicin in a dose-dependent manner, followed by a reduction of total protein. In addition, a well-known protein synthesis inhibitor, cycloheximide, also decreased the apical enzyme activities. These results suggest that the inhibition of protein synthesis by gentamicin is a possible cause of the decreased activities of apical membrane enzymes in LLC-$PK_1$ cells. The inhibition of protein synthesis may be related to the nephrotoxicity induced by aminoglycoside antibiotics.**

**Keywords** gentamicin; aminoglycoside antibiotics; kidney; nephrotoxicity; LLC-$PK_1$; apical membrane enzyme; protein synthesis; cycloheximide

## Introduction

Aminoglycoside antibiotics are polycationic molecules that inhibit bacterial protein synthesis, and are widely used in the treatment of gram-negative infectious diseases.[1] However, their use is sometimes associated with nephrotoxicity, as evidenced by defective urine concentrating capacity, proteinuria, enzymuria, and tubular cell necrosis.[2] The mechanisms of nephrotoxicity induced by aminoglycoside antibiotics have been extensively studied, and it was found that many structural and functional alterations occurred in lysosomes, plasma membranes, and mitochondria during and/or after the development of nephrotoxicity.[3,4] However, the biochemical events responsible for proximal tubular injury and subsequent renal failure are still unclear.

Cell culture is a powerful tool with which to study the biochemical mechanisms underlying the cytotoxicity of drugs. LLC-$PK_1$ is a continuous cell line derived from the pig kidney, and possesses many similarities to the proximal tubular epithelium. The LLC-$PK_1$ cells form an oriented monolayer with microvilli at their apical side, tight junctions, and exhibit unidirectional transport of salt and water, resulting in dome formation.[5] They also possess apical membrane enzymes and transport systems for hexose,[6-11] amino acids,[12] phosphate,[13] and organic cations[14-16] similar to those observed in the proximal tubules. Because tubular cell necrosis induced by aminoglycosides is confined almost exclusively to the proximal tubules, LLC-$PK_1$ cells should represent a good model system to study the cytotoxicity of these antibiotics. We and several other investigators have used LLC-$PK_1$ cells to study the mechanisms of cellular toxicity of nephrotoxic drugs such as aminoglycosides,[17-21] *cis*-diamminedichloroplatinum(II),[22] and cyclosporin.[23]

We have previously shown in LLC-$PK_1$ cells that apical membrane enzyme activities were inhibited by aminoglycoside antibiotics.[20] In addition, the potency of various aminoglycosides to inhibit the enzyme activities was in the decreasing order of neomycin, gentamicin, sisomicin, tobramycin, netilmicin, amikacin, kanamycin, and astromicin, and their order was correlated to their nephrotoxic potentials *in vivo*. Because gentamicin did not directly inhibit the apical membrane enzyme activities when added to LLC-$PK_1$ cell homogenates,[17] the decreased enzyme activities were presumably indirectly induced by interference with some intracellular processes. This background prompted us to study the mechanisms of the decreased apical membrane enzyme activities by gentamicin treatment in LLC-$PK_1$ cells, which may help to reveal the initial event responsible for aminoglycoside nephrotoxicity. The results indicated that the inhibition of protein synthesis by gentamicin is, at least in part, related to a decrease in the enzyme activities in LLC-$PK_1$ cells.

## Materials and Methods

**Cell Culture** LLC-$PK_1$ cells obtained from the American Type Culture Collection (ATCC CRL-1392) were grown on plastic dishes (Corning Glass Works, Corning, NY) in medium 199 (Flow Laboratories, Rockville, MD), supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD) without antibiotics, in an atmosphere of 5% $CO_2$–95% air at 37 °C. The cells were subcultured every 4—5 d using 0.02% EDTA and 0.05% trypsin. In most experiments, 60 and 100 mm dishes as well as six-well plates were seeded with $4 \times 10^5$ cells in 5 ml, $1 \times 10^6$ cells in 10 ml and $2 \times 10^5$ cells in 2 ml of complete culture medium (medium 199 supplemented with 10% fetal bovine serum), respectively. The cells used here were between passages 217 and 233.

**Enzyme Assays** LLC-$PK_1$ cells were washed twice with ice-cold saline, then removed with a rubber policeman into ice-cold saline (2 ml for 60 mm dish, and 4 ml for 100 mm dish). The cells were homogenized with a polytron (Kinematica, Kriens-Luzern, Switzerland) at a setting of 7 for 1 min or with a bath-type sonicator (model G112SP1, Laboratory Supplies, Hicksville, NY) three times for 5 s each. Alkaline phosphatase (EC 3.1.3.1), aminopeptidase (EC 3.4.11.2), and $\gamma$-glutamyltransferase (EC 2.3.2.2) in the homogenate were measured as described previously.[17,20] Protein was measured by the method of Bradford,[24] using the Bio-rad protein assay kit with bovine $\gamma$-globulin as the standard.

**Measurement of the Rate of Protein Synthesis** [$^3$H]Leucine incorporation was measured in LLC-$PK_1$ cells grown in six-well, cluster plates. After seeding, the plates were left for two hours in a $CO_2$ incubator, then the culture medium was replaced with that containing gentamicin. At the stated times after gentamicin addition, cells were rinsed twice with Dulbecco's phosphate buffered saline (PBS buffer; 137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). The cells were then incubated with 1 ml of serum-free medium 199 containing [$^3$H]leucine (1 $\mu$Ci/ml) for 30 min at 37 °C in a $CO_2$ incubator. The

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medium was immediately aspirated and the wells were rapidly rinsed three times with ice-cold PBS buffer. The cells were fixed with 1 ml of 2.5% trichloroacetic acid for 10 min, then rinsed twice with 2 ml of 2.5% trichloroacetic acid followed by one rinse with 2 ml of ethanol. The residues were solubilized with 0.1 N NaOH, and aliquots were transferred into scintillation vials for counting [$^3$H]leucine incorporation. Protein was measured as described above.

**Materials** Gentamicin sulfate and cycloheximide were obtained from Sigma Chemicals Co. (St. Louis, MO) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. L-[$^3$H]Leucine (140 Ci/mmol) was obtained from Amersham International, Ltd. (Buckinghamshire, UK). All other chemicals were of the highest purity available.

## Results

To determine the relationship between the developing status of apical membrane enzyme activities and the effect of gentamicin on these enzymes, LLC-PK$_1$ cells were incubated with gentamicin for 2 d at various times after seeding. Thereafter, the specific activity of the enzyme was measured. As shown in Fig. 1, the activity of alkaline phosphatase in control cells increased during growth in culture up to day 7, then decreased. Alkaline phosphatase activity was markedly inhibited by gentamicin when the enzyme activity was increasing (days 3—5, 5—7). On the other hand, the effect of gentamicin was less pronounced when the enzyme activity was decreasing (day 7—9) or relatively constant (day 9—11). The effect of gentamicin on other apical membrane enzymes, aminopeptidase and $\gamma$-glutamyltransferase, was also studied (Fig. 2). Again, gentamicin inhibited these enzymes when the activities were increasing but the effect was marginal when the activities were decreasing or relatively constant.

Alkaline phosphatase activity in the homogenate of control and gentamicin treated cells was measured at various substrate concentrations (Fig. 3). LLC-PK$_1$ cells were treated with either 2 or 5 mM gentamicin for 4 d. The alkaline phosphatase activity in gentamicin treated cells was lower than that of control cells at all substrate concentrations, and it is apparent that the $V_{max}$ (the maximal enzyme activity) of alkaline phosphatase was significantly decreased by gentamicin in a dose-dependent manner. Eadie–Hofstee analysis of the data showed that $V_{max}$ values of alkaline phosphatase in the control, 2, and 5 mM gentamicin-treated cells were 35.9, 27.5, and 12.9 nmol/mg protein/min, respectively. On the other hand, the $K_m$ (the apparent affinity) values were less affected by gentamicin (10, 10, and 7 $\mu$M in the control, 2, and 5 mM gentamicin-treated cells, respectively). Similar effects of gentamicin on aminopeptidase were observed, and the $V_{max}$ value of the enzyme was decreased by 31.8% ($n=3$) by the treatment of 2 mM

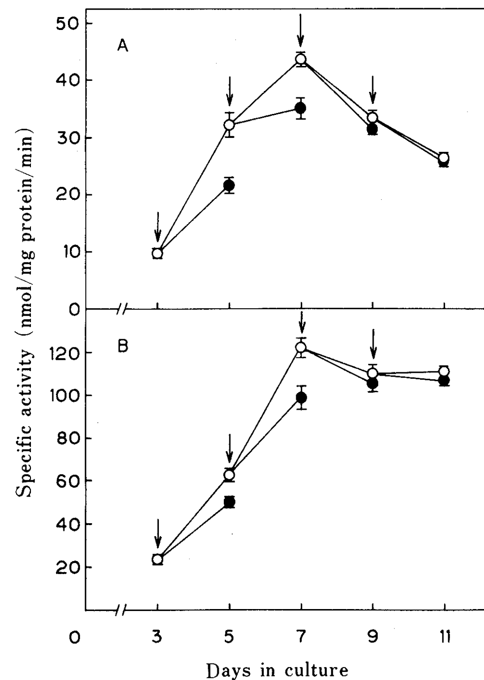


Fig. 2. Effect of Gentamicin on Aminopeptidase and $\gamma$-Glutamyltransferase Activities

LLC-PK$_1$ cells were treated with gentamicin as described in Fig. 1, and the specific activities of aminopeptidase (A) and $\gamma$-glutamyltransferase (B) were measured. ○, control; ●, 2 mM gentamicin. Each point represents the mean ± S.E. of six determinations from two separate experiments.

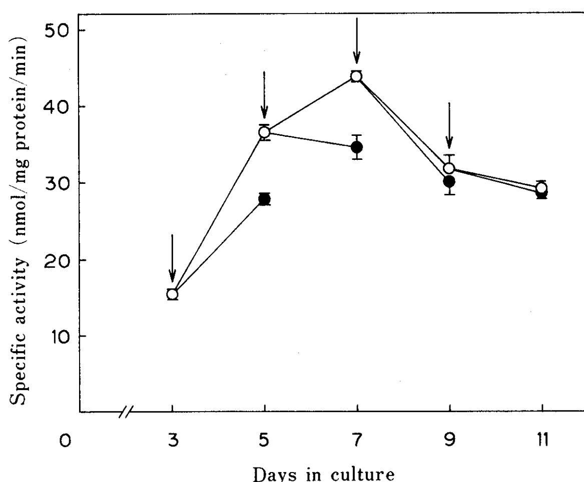


Fig. 1. Effect of Gentamicin on Alkaline Phosphatase Activity in LLC-PK$_1$ Cells

LLC-PK$_1$ cells were cultured in plastic dishes. At days 3, 5, 7 and 9 after seeding (indicated by arrow), the culture medium was replaced with fresh medium without or with 2 mM gentamicin. After 2 d of incubation, the specific activity of alkaline phosphatase in homogenate of control (○) and gentamicin treated cells (●) was determined. Each point represents the mean ± S.E. of six determinations from two separate experiments.

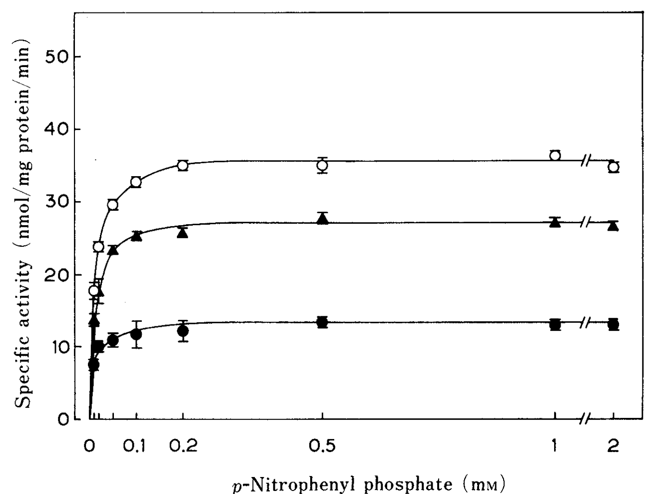


Fig. 3. Effect of Gentamicin on the Maximum Activity of Alkaline Phosphatase

LLC-PK$_1$ cells were incubated with gentamicin on the day of seeding. After 4 d, the activity of alkaline phosphatase in control (○), 2 mM (▲), and 5 mM (●) gentamicin treated cells was determined at various concentrations of substrate ($p$-nitrophenyl phosphate). Each point represents the mean ± S.E. of three determinations from a typical experiment.

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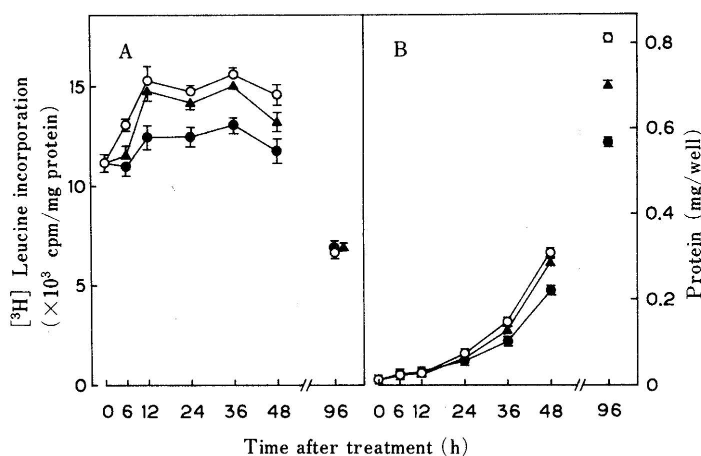


Fig. 4. Effect of Gentamicin on [$^3$H]Leucine Incorporation and Cell Protein

LLC-PK$_1$ cells were seeded into six-well cluster plates. After inoculation, the plates were left in a $CO_2$ incubator for 2 h, then the culture medium was replaced with fresh medium with or without gentamicin. At various times thereafter, [$^3$H]leucine incorporation (A) and the total amount of cell protein per well (B) were determined in control (○), 2 mM (▲), and 5 mM (●) gentamicin treated cells. Each point represents the mean ± S.E. of six or seven determinations from two separate experiments.

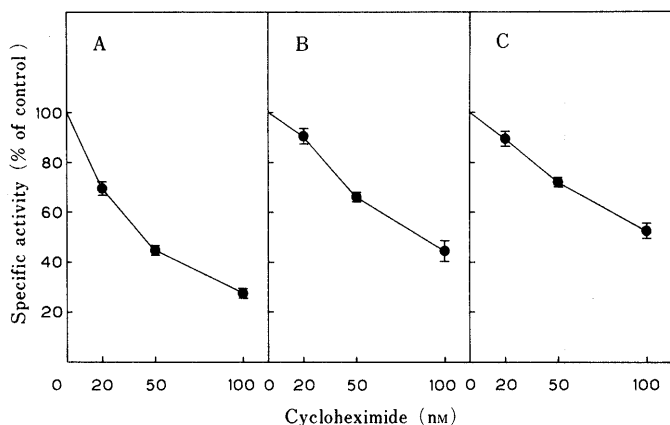


Fig. 5. Effect of Cycloheximide on Apical Membrane Enzyme Activities

LLC-PK$_1$ cells were incubated with various concentrations of cycloheximide on the day of seeding. After 4 d, the specific activities of alkaline phosphatase (A), aminopeptidase (B), and γ-glutamyltransferase (C) activities were determined. Each point represents the mean ± S.E. of six determinations from two separate experiments.

gentamicin.

The effect of gentamicin on protein synthesis was evaluated in LLC-PK$_1$ cells. The cells were incubated with gentamicin, then the level of [$^3$H]leucine incorporation and the amount of cell protein per well were measured at various times. As shown in Fig. 4A, [$^3$H]leucine incorporation in the control cells increased after seeding, then was nearly constant. At confluence (96 h), [$^3$H]leucine incorporation decreased markedly. Gentamicin inhibited [$^3$H]leucine incorporation after 6 h. The inhibitory effect of gentamicin on [$^3$H]leucine incorporation was dose dependent. At confluence, however, inhibition was not observed. Protein concentrations in the control and gentamicin treated cells were similar up to 12 h, but thereafter gentamicin suppressed the increase of cell protein in a dose-dependent manner (Fig. 4B). In contrast to the effect on [$^3$H]leucine incorporation, the decrease in the protein concentration induced by gentamicin was apparent at confluence, and was greater than at earlier time points. Thus, gentamicin inhibited protein synthesis in LLC-PK$_1$ cells, followed by a decrease of cellular protein concentrations.

The effect of the protein synthesis inhibitor, cycloheximide, on apical membrane enzyme activities was investigated. LLC-PK$_1$ cells were incubated with various concentrations of cycloheximide for 4 d, then the activities of alkaline phosphatase, aminopeptidase and γ-glutamyltransferase were measured. As shown in Fig. 5, the specific activities of these enzymes were dose dependently decreased by cycloheximide.

## Discussion

Aminoglycoside antibiotics induce various functional as well as structural alterations in the renal proximal tubules. In LLC-PK$_1$, an established cell line with similar characteristics to those of proximal tubular epithelial cells, one of the manifestations of gentamicin cytotoxicity is the decreased activity of marker enzymes of the apical membrane.[17,20] In addition, the relative toxicities of various aminoglycosides for the apical membrane enzymes are, in part, compatible with their nephrotoxicities *in vivo*.[20] The decreased activity of renal brush-border membrane enzymes was also reported in rats injected with gentamicin.[3,25,26] Thus, it is of merit to study the mechanisms related to decreased apical membrane enzyme activity, for a better understanding of the molecular events responsible for gentamicin nephrotoxicity. In the present report, we further studied the effect of gentamicin on apical membrane enzyme activities in LLC-PK$_1$ cells. The results demonstrated that the decreased activities of apical membrane enzymes were related to the inhibition of protein synthesis by gentamicin.

In LLC-PK$_1$ cells, apical membrane enzyme activities develop concurrently with the differentiation of the cells on and after the formation of tight junctions.[27-29] In our experiments, the activities of alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase increased up to day 7 after seeding, then decreased or were unchanged (Figs. 1 and 2). The inhibitory effect of gentamicin on these enzymes was more pronounced during the former than during the latter period (Figs. 1 and 2). The results thus indicate that the inhibition of apical membrane enzymes by gentamicin is not due to the direct interaction of the drug with the enzymes or with the apical membrane. Up to 7 d after seeding, the rate of synthesis of apical membrane enzyme molecules should exceed that of degradation, and at the latter period, the relationship should be reversed. Therefore, it is likely that gentamicin exerted its inhibitory effect by reducing the synthesis of active enzyme molecules. The finding that gentamicin decreased the maximal activity but not the affinity of apical membrane enzyme (Fig. 3) supports this notion. We previously reported that the inhibitory effect of gentamicin on the activity of $(Na^+ + K^+)$-ATPase, a marker enzyme for basolateral membrane, was relatively small compared with that on apical membrane enzyme activities.[20] This may be related to the fact that the activity of $(Na^+ + K^+)$-ATPase did not change appreciably during the culture period.

Gentamicin kills bacteria by inhibiting protein synthesis through failure of initiation and misreading of messenger RNA.[1,30] Aminoglycoside antibiotics also inhibit eukaryotic microsomal protein synthesis.[31-33] Buss and Piatt[34]

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reported that gentamicin administered *in vivo* reduced protein synthesis in microsomes isolated from rat kidney. Subsequently, Bennett *et al.*[35] demonstrated that [$^3$H]leucine incorporation into renal microsomes isolated from rats injected with gentamicin was significantly reduced, even though the morphology and functions of the kidney were well preserved. In the present study, gentamicin inhibited [$^3$H]leucine incorporation in LLC-$PK_1$ cells except at 96 h after treatment (Fig. 4). At that time, LLC-$PK_1$ cells reached confluence and therefore, the rate of total protein synthesis in control cells, which was assessed by [$^3$H]leucine incorporation, decreased (Fig. 4A). In contrast, the apical membrane enzyme activity increased even after confluence was achieved, and was inhibited by gentamicin (Figs. 1 and 2, days 5—7). Thus, under the above experimental conditions, the inhibitory effect of gentamicin on apical membrane enzymes could be detected more sensitively than that on total protein synthesis.

The inhibition of protein synthesis should be related to decreased apical membrane enzyme activities, as described above. The correlation between the decreased specific activities of apical membrane enzymes and the inhibition of protein synthesis was also supported by a study using cycloheximide, a protein synthesis inhibitor (Fig. 5). We previously postulated that aminoglycoside-induced alterations of apical membrane functions in LLC-$PK_1$ cells are related to the increase in cytosolic free calcium concentration.[20] The concentration of cytosolic free calcium was elevated when LLC-$PK_1$ cells were treated with gentamicin for 2 d. On the other hand, the inhibitory effect of gentamicin on protein synthesis was observed even after 6 h (Fig. 4). In addition, protein synthesis was inhibited prior to the decrease of apical membrane enzyme activities, because incubation of LLC-$PK_1$ cells with gentamicin for 6 h did not affect the specific activities of alkaline phosphatase and aminopeptidase (data not shown). Therefore, though gentamicin has multiple effects on the cellular functions, the inhibition of protein synthesis may represent an early manifestation of its toxicity. This finding is consistent with that reported by Bennett *et al.*[35]

Gentamicin accumulates within the kidney, and almost exclusively in the proximal tubules.[2] Though the mechanism of gentamicin uptake by the renal proximal tubular cells is not yet fully understood, it is generally accepted that the process involves binding to brush-border membranes, endocytic uptake and transfer to lysosomes.[36,37] At this point, it is unclear how gentamicin could gain access to cytosol, and subsequently inhibit protein synthesis. It may enter the cytosol either by "spillover" from lysosomal storage sites[38] or *via* another transport process. Buchanan *et al.*[39] reported that G418, an aminoglycoside antibiotic with a structure closely related to gentamicin, is taken up by endocytosis in human fibroblasts, and inhibits the rate of [$^{35}$S]methionine incorporation. Thus, as they concluded, aminoglycoside antibiotics such as gentamicin and G418 could reach the ribosomes. Further studies are needed to clarify the intracellular handling of the antibiotics.

In conclusion, our findings indicate that the gentamicin-induced decrease of apical membrane enzyme activities is, at least in part, due to the inhibition of protein synthesis in LLC-$PK_1$ cells. The inhibition of protein synthesis may be related to the nephrotoxicity induced by aminoglycoside antibiotics.

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