MODE OF PROTECTIVE ACTION OF FOSFOMYCIN AGAINST DIBEKACIN-INDUCED NEPHROTOXICITY IN THE DEHYDRATED RATS

SHIGEHARU INOUE, TETSUTARO NIIZATO, IZUMI KOMIYA, YASUKATSU YUDA AND YUJIRO YAMADA

Central Research Laboratories, Meiji Seika Kaisha, Ltd., Morooka, Kohoku-ku, Yokohama 222, Japan

(Received April 9, 1982)

We studied the mechanism on protective effect of fosfomycin against experimental nephrotoxicity induced by dibekacin. In order to simplify an experimental model, the dehydrated Wistar rats were used, because a single injection of dibekacin at 30 mg/kg induced acute renal failure in the dehydrated rats, characterized by alteration of urinalytic parameters and BUN values, and retarded elimination of dibekacin from blood. When the rats were administered simultaneously with fosfomycin at 120 mg/kg, the rate of elimination was restored almost to normal, accompanied with improvement of the nephrotoxic parameters. However, markedly accelerated elimination over normal one was not observed, indicating that the improved elimination was not the reason of protection but a result of normal kidney function. On the other hand, fosfomycin protected the proximal tubular lysosomes from the injury of aminoglycoside, as evidenced a) *in vivo* by suppression of myeloid body formation and protection of lysosomal membrane integrity of the rats treated with dibekacin, and b) *in vitro* by dose-dependent protection of the lysosomal membrane integrity of the kidney cells. A study of structure-protective activity relation revealed that phosphonate anion possessing an epoxy function was important for protection, and that the mechanism of protection differed from the antibacterial mechanism.

Keywords — antibiotic; fosfomycin; fosfomycin analogs; dibekacin; nephrotoxicity; urinalysis; drug elimination; kidney lysosome; dehydrated rat

INTRODUCTION

In the preceding paper,¹⁾ we reported that a broad spectrum antibiotic fosfomycin showed the capability to reduce the experimental nephrotoxicity induced by an aminoglycoside dibekacin, when administered concurrently to the Fischer rats with free access to water. Protective effect of fosfomycin was shown by urinalysis, serum analysis and histo-pathological examination of proximal tubules. However, in order to confirm the protective activity, it was required to clarify the mechanism of protection, which is inevitably related to the mechanism of aminoglycoside induced nephrotoxicity. Many workers have indicated that aminoglycoside induced nephrotoxicity was closely associated with the selective and long-lasting accumulation in the kidneys,²⁾ in particular in lysosomes of

proximal tubular cells,³⁾ and that this accumulation induced biochemical disorder and eventually lesion.⁴⁾

Therefore, two approaches have been examined in this investigation for the mode of protective action, *i.e.*, pharmacokinetic and biochemical approaches using the dehydrated Wistar rats. In the former, the effect of fosfomycin was examined on the excretion of dibekacin, and in the latter the effect on the lysosomal morphology and membrane integrity affected by dibekacin. Finally, the relationship between structure and protective activity of the fosfomycin analogs was studied, seeking the structural requirement for the protective action.

MATERIALS AND METHODS

Drugs — Fosfomycin sodium and dibekacin

sulfate were products of Meiji Seika Kaisha, Ltd., Tokyo, Japan. Tritium-labeled dibekacin was prepared in our laboratories by catalytic reduction of 3',4'-dehydrodibekacin in the presence of tritium gas. Specific activity of dibekacin- ^3H was $138\ \mu\text{Ci}/\text{mg}$. Inulincarboxyl- ^{14}C with specific activity of $1.93\ \mu\text{Ci}/\text{mg}$ was purchased from New England Nuclear Co., Boston, USA. Sodium (+)-(1*S*, 2*R*)-epoxypropylphosphonate was obtained by optical resolution of a synthetic racemate of (+, -)-epoxyisomers, and sodium (1*R*, 2*R*)-1,2-dihydroxypropylphosphonate was prepared from fosfomycin by treating with 1*N* hydrochloric acid. Sodium phosphonoformate and phosphorylethanolamine were supplied from Sigma Chemical Company, Saint Louis, USA. A drug or a mixture of drugs was dissolved in distilled water, and injected intravenously by 0.5 ml. The control groups received 0.5 ml saline.

Determination of Urinary and Serum Parameters of Nephrotoxicity — Male Wistar SPF rats, 7–8 weeks old, were fed with commercial rat chow NMF (Oriental Kōbo Ltd., Tokyo) without water for 48 h before treatment. Dehydration was continued further for 17 h, and thereafter animals were allowed access to water, and were housed in metabolic cages to collect urine sample for 24 h. Urinary parameters employed in this study were urinary volume, osmolality, protein content, the number of nucleated cells, *N*-acetyl- β -D-glucosaminidase (NAG) activity and lactate dehydrogenase (LDH) activity. Osmolality was determined with an Advanced osmometer, and protein content was measured by the colorimetry with sulfosalicylic acid⁵⁾ and was expressed as mg per 24-h urine. The number of nucleated cells was counted under a light microscope on the precipitate obtained by centrifuging the urine sample at 1500 rpm for 5 min.¹⁾ NAG and LDH activities were determined by spectrophotometry, using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide⁶⁾ and pyruvic acid⁷⁾ as the respective substrates, and were expressed as International Unit (IU) per 24-h urine.

Serum and kidney samples were obtained from the rats after collection of urine sample was completed, *i.e.*, 41 h after administration of drugs. Blood urea nitrogen (BUN), serum creatinine, relative kidney weight (kidney wt./body wt. $\times 100$) and renal histo-pathology by light and electron microscopes were examined. BUN and serum creatinine were measured with a Hitachi Model 716 autoanalyzer. Histo-pathological studies were performed according to the procedure described in the preceding paper.¹⁾ The ultrastructural study was made with a Hitachi H-700 electron microscope, after the samples were fixed in solution of 2.5% glutaraldehyde in 1*M* phosphate buffer (pH 7.2). Post-fixation was made in 2% osmium tetroxide for one hour. The slices were stained with uranyl acetate and lead citrate.

Measurement of Plasma, Urinary and Renal Concentrations of Dibekacin — Male Wistar SPF rats, 7–8 weeks old, were dehydrated 48 h before treatment, and dehydration was maintained during the experiments. Before the administration of drugs, the animals were anesthetized by the intraperitoneal injection of sodium pentobarbital (50 mg/kg), and polyethylene tubings were cannulated through the femoral artery and the bladder for sampling of blood and urine, respectively. The animals were fixed on the operation boards during the blood sampling, and thereafter were kept in metabolic cages.

About 0.3 ml of blood was taken at each measurement, and the heparinized blood was centrifuged at 3000 rpm for 10 min to obtain plasma specimen. Blood and urine specimens were obtained eight times from the same animal. Twenty-four hours after treatment, animals were exsanguinated, and renal cortices were obtained. Wistar rats with free access to water throughout the experiments were used as reference groups. One hundred microliters of plasma and urine or 0.2 g of renal cortex were subjected to a sample oxidizer (Packard Co., Model 306), and the radioactivity was measured with a liquid scintillation counter (Packard Co., Model 3380). Pharmacokinetic parameters were calculated on

the basis of two-compartment model.

Assay of Lysosomal Membrane Integrity

—Sixty male Wistar rats, with an average weight of 400 g, were sacrificed by exsanguination. Kidneys were immediately flushed with 0.25M sucrose to remove blood, then suspended in five volumes of 0.25M sucrose at 4°C, and homogenized with a Teflon homogenizer. The crude homogenate was centrifuged at 2100 rpm for 15 min at 5°C, and the supernatant was centrifuged at 23500 rpm for 20 min at 2°C by gradient sedimentation over 20–70% (w/v) sucrose. The lysosome-rich fraction was identified by the measurement of the acid phosphatase activity.

To the lysosome-rich fraction suspended in 0.25M sucrose, a drug dissolved in 0.25M sucrose was added, and the mixture was incubated at 37°C for 1 h. The sample was cooled, and centrifuged at 12000 rpm for 0.5 h at 2°C. The amount of acid phosphatase liberated in the supernatant was assayed using β -glycerophosphate as a substrate. The results were expressed in percentage release of the acid phosphatase activity of the lysosomal fraction of control rats after thermal shock (37°C for 30 min). Using the same procedure, the lysosomal fractions were prepared from the dehydrated rats receiving dibekacin or dibekacin plus fosfomycin in ad-

vance, and acid phosphatase released by thermal shock at 37°C was assayed without addition of drugs.

RESULTS

Protective Effect of Fosfomycin against Dibekacin-induced Nephrotoxicity in the Dehydrated Rats

Protective effect of fosfomycin on the dibekacin-induced nephrotoxicity in the dehydrated Wistar rats is shown in Table I. Upon one injection of 30 mg/kg of dibekacin, the values of urinary volume, protein content, the number of nucleated cells and NAG were increased to two to three times the non-drug control. The most remarkable was LDH, a typical cytosol enzyme, which was elevated more than twenty times the control. Urinary osmolality decreased to two-third of the control. When dibekacin was injected in combination with fosfomycin, the values of the six parameters were considerably improved. Of these, urinary volume, osmolality, protein, NAG and LDH were restored to the normal level, while the number of nucleated cells decreased to half that of the dibekacin alone. The protective effect of fosfomycin was also seen on the serum analysis. BUN values remained in the normal range in the group of dibekacin-fosfomycin combination, while BUN values were elevated two times the

TABLE I. *Protective Effect of Fosfomycin from Dibekacin induced Nephrotoxicity in the Dehydrated Rats: Correlation of Urinalysis, Serum Analysis and Lysosomal Membrane Integrity*

	Dose (mg/kg)	Urinalysis						Serum analysis		Release of acid phosphatase IU/g	n
		Volume ml/24h	Osmolality mosm/kg	Protein mg/24h	Nucleated cells/24h	NAG mIU/24h	LDH mIU/24h	BUN mg/dl	Creatinine mg/dl		
Non-drug control	0	4.4 ±1.7	2625 ± 282	168 ± 73	6850 ±1790	117 ± 59	100 ± 5	24.8 ±0.72	0.93 ±0.15	23.6 ± 0.61	3
Dibekacin	30	7.1 ±2.0	1932 ± 675	431↑ ±137	22200↑ ±5300	258↑ ± 63	2360↑ ±154	52.6↑ ±10.0	0.97 ±0.12	30.7↑ ± 0.88	3
Dibekacin plus fosfomycin	30 120	4.2 ±1.2	2808 ± 501	224 ±124	10900↑ ±1400	110 ± 12	100 ± 3	24.1 ± 1.0	0.93 ±0.06	26.8↑ ± 0.97	3

Results are expressed as mean ± standard deviation.

↑ significantly different from the non-drug control ($p < 0.05$).

non-drug control in a case of dibekacin alone. Creatinine was unaffected under the experimental conditions employed. Thus, the protective action of fosfomycin against dibekacin-induced nephrotoxicity was confirmed using the dehydrated Wistar rats.

Effect of Fosfomycin on the Pharmacokinetics of Dibekacin

After dibekacin- ^3H was administered at doses of 30 mg/kg and 0.56 mg/kg into rats, with or without fosfomycin (120 mg/kg), plasma con-

centration, urinary recovery and renal cortical accumulation of dibekacin were examined.

a) Plasma Elimination — Fig. 1 shows the plasma concentration *versus* time of dibekacin. The dehydrated rats receiving dibekacin alone (30 mg/kg) showed the most retarded elimination ($T_{1/2}$ (β) 83 min), while the dehydrated rats receiving dibekacin (30 mg/kg) plus fosfomycin (120 mg/kg) showed $T_{1/2}$ (β) 52 min. The latter was almost equivalent to those of the water-supplied rats ($T_{1/2}$ (β) 48 min for single and 40 min for combination). Thus, fosfomycin showed a remarkable effect on the plasma half-life of dibekacin in the dehydrated rats, while no effect was recognized in the rats with free access to water.

This was supported by examining glomerular filtration rate (GFR) of inulin- ^{14}C administered concurrently with dibekacin and fosfomycin. As summarized in Table II, elimination rate of inulin- ^{14}C was low only when the dehydrated rats were given dibekacin alone, and the other three groups showed normal elimination rate. When the dose of dibekacin was reduced to 0.56 mg/kg, dibekacin did not cause renal toxicity even in the dehydrated rats. The plasma half-life ($T_{1/2}$ (β) 44 min) of dibekacin alone at this dosage was similar to that ($T_{1/2}$ (β) 35 min) of dibekacin associated with fosfomycin in the dehydrated rats (Fig. 1). An important finding was that fosfomycin did not cause significant acceleration in the elimination of dibekacin beyond normal rate, even in the absence of nephrotoxicity.

b) Urinary Recovery — Urinary excretion of dibekacin in the four groups of rats receiving 30 mg/kg of dibekacin with or without 120 mg/kg of fosfomycin is illustrated in Fig. 2. Cumulative urinary recovery of dibekacin was 66% in 24 h in the dehydrated group receiving dibekacin alone, but it was improved to 81% by the addition of fosfomycin. The latter recovery was comparable to that of the two water-supplied groups (83% both for single and combination). Again, the two groups supplied with water revealed no difference in urinary recovery, indicating no

FIG. 1. Effect of Fosfomycin (120 mg/kg) on Plasma Concentration of Dibekacin after Intravenous Administration in the Dehydrated and Water-supplied Rats

$\blacktriangle$ Dibekacin alone in dehydrated rats, $\triangle$ dibekacin plus fosfomycin in dehydrated rats, $\bullet$ dibekacin alone in water-supplied rats, $\circ$ dibekacin plus fosfomycin in water-supplied rats. Values represent mean $\pm$ standard deviation.

TABLE II. *Effect of Fosfomycin on the Pharmacokinetic Parameters of Inulin-¹⁴C in the Dehydrated and Water-supplied Rats bearing Dibekacin*

Animal	Dose (mg/kg)		Plasma elimination			Urinary recovery (%)	
	Dibekacin	Fosfomycin	V_{dss} (l/kg)	β (min ⁻¹)	$T_{1/2} \beta$ (min)	3 h	24 h
Water-supplied rats	30	0	0.243	0.0247	28.1	88	93
Water-supplied rats	30	120	0.307	0.0270	25.7	88	96
Dehydrated rats	30	0	0.268	0.0121	57.3	66	88
Dehydrated rats	30	120	0.261	0.0244	28.4	85	88

Results are expressed as mean $\pm$ standard deviation.

FIG. 2. *Effect of Fosfomycin (120 mg/kg) on Urinary Excretion of Dibekacin (30 mg/kg), after Intravenous Administration in the Dehydrated and Water-supplied Rats*

▲ Dibekacin alone in dehydrated rats, △ dibekacin plus fosfomycin in dehydrated rats, ● dibekacin alone in water-supplied rats, ○ dibekacin plus fosfomycin in water-supplied rats. Values represent mean $\pm$ standard deviation.

effect of fosfomycin.

c) *Renal Accumulation* — Renal cortical accumulation of dibekacin after intravenous administration is shown in Table III. Among the four groups receiving 30 mg/kg of dibekacin, the most heavy accumulation of dibekacin was observed in the dehydrated rats given dibekacin alone. In contrast, no effect of fosfomycin was seen in the water-supplied rats. When the dose was reduced to 0.56 mg/kg, no difference in the

renal accumulation was observed in the dehydrated rats given dibekacin alone or with fosfomycin.

In Vivo and in Vitro Effect of Fosfomycin on the Lysosomal Membrane Lability induced by Dibekacin

We have examined the effect of fosfomycin on the renal lysosomes in the following three respects:

a) *Effect of Fosfomycin on the Myeloid Body Formation* — Formation of myeloid bodies in the kidney lysosomes after aminoglycoside administration is well known.⁸⁾ As illustrated in Fig. 3, ultrastructural investigation on the proximal tubular cells revealed that myeloid bodies formed within the lysosomes were enlarged in the dehydrated rats receiving dibekacin alone, but they remained small when fosfomycin was administered concurrently. Furthermore, the size of lysosomes themselves was near normal in the combination group, whereas, the swollen secondary lysosomes were more prominent in the rats treated with dibekacin alone. It should be noted, however, that no histo-pathological damage was detected under light microscopy even in the kidney specimens from rats receiving dibekacin only.

b) *In Vivo Protection of Fosfomycin on the Lysosomal Membrane Integrity* — The lysosome-rich fraction was freshly prepared from the dehydrated rats given 30 mg/kg of dibekacin,

with or without fosfomycin (120 mg/kg), and the lysosomal membrane integrity was estimated by measuring the quantity of acid phosphatase released, a marker enzyme of lysosome, upon thermal shock. As listed in Table I, the lysosomal fraction of dibekacin alone indicated a slight but significant increase of the free enzyme activity on thermal shock, corresponding to the enhancement of membrane lability. Concomitant administration of fosfomycin reduced the leakage of the marker enzyme, coincidental with the protective action of fosfomycin.

c) In Vitro Effect of Fosfomycin on the Lysosomal Membrane in the Presence of Dibekacin

—The renal lysosome-rich fraction was prepared from the non-treated rats, and the membrane integrity challenged by thermal shock was checked out under various concentrations of dibekacin and fosfomycin. The results are shown in Fig. 4. Dibekacin alone caused steady increase in release of acid phosphatase in parallel to the increasing drug concentration in a range from 250 to 1500 mg/l. Addition of fosfomycin to the constant concentration of dibekacin at 1500 mg/l showed suppression of the enzyme release in proportion to the increasing concentration of

fosfomycin added, and the stabilizing effect of fosfomycin was significant at 1000 and 1500 mg/l. Fosfomycin alone gave a slight suppression of the release of acid phosphatase under this condition.

Protective Activity of Fosfomycin Analogs

Table IV shows the protective activity of five analogs of fosfomycin. All of the analogs have phosphonic acid function, and the activity was compared by using BUN, serum creatinine and relative kidney weight as nephrotoxic parameters. (+)-Epoxypropylphosphonate, an optical isomer of fosfomycin, exhibited protective activity comparable to fosfomycin. 1,2-Dihydroxypropylphosphonate and *cis*-propenylphosphonate, which have the same carbon skeleton as fosfomycin but lack of epoxy group, were weaker in protective action than fosfomycin. Sodium phosphonoformate containing three molecules of sodium cation was weaker than fosfomycin containing two molecules of sodium cation. Phosphorylethanolamine that does not possess sodium cation was inactive.

DISCUSSION

Recently, the use of high dose of aminoglyco-

TABLE III. *Effect of Fosfomycin on Renal Cortical Concentration of Dibekacin, after Intravenous Administration in the Dehydrated and Water-supplied Rats*

Animal	Dose (mg/kg)		Renal cortical concentration ($\mu\text{g/g-tissue}$)	n
	Dibekacin	Fosfomycin		
Water-supplied rats	30	0	367 ± 21	4
	30	120	270 ± 45	4
Dehydrated rats	30	0	690 ± 388	3
	30	120	348 ± 58	3
Dehydrated rats	0.56	0	25.9 ± 4.8	4
	0.56	120	30.0 ± 9.5	4

Results are expressed as mean $\pm$ standard deviation.

FIG. 3. Electron Micrographs of the Proximal Tubular Cells from the Dehydrated Rats treated with Dibekacin (30 mg/kg) alone (I) or plus Fosfomycin (120 mg/kg) (II)

An increased number of the large myeloid bodies (MB) is seen in the swollen lysosomes (L). Brush border microvilli (BB), mitochondria (M) and nuclei (N) are normal (Plate I). The proximal tubular cells appear normal except for small myeloid bodies within lysosomes of normal size (Plate II). 5000 $\times$.

side many times greater than the clinical dosage was questioned in the comparative nephrotoxic study in rats.⁹⁾ The dosage could be reduced either by repeated administration to rats with free access to water¹¹⁾ or by imposing burden to kidney by dehydration¹⁰⁾ and addition of plasma expanders⁸⁾ or diuretics.¹¹⁾ Of these, the dehydrated model using Wistar rats has been well established and used in the protection experiments against aminoglycoside-induced nephrotoxicity.^{12,13)} The dehydrated model has an advantage in that a single injection of dibekacin at 30 mg/kg was adequate to induce acute renal failure. This made it possible to analyze the combination effect of fosfomycin with good reproducibility. In contrast, a single injection of 40 mg/kg of dibekacin into the rats with free access to water caused little sign of nephrotoxicity, and its repeated administrations were required to induce the nephrotoxicity, even with highly sensitive Fischer 344 rats.¹⁾ When the two toxicity models were compared, the acute model showed a drastic change of the uranalytic parameters and BUN value. But the two models shared common features of polyuria, proteinuria, cysturia, enzymuria, azotemia and formation of myeloid bodies. Thus, although the subacute model should be closer to nephrotoxic symptom in clinical situation than the acute model, use of the simplified acute model may be applied for the first step to study the mechanism of protection by fosfomycin.

The mechanism of the protective action of fosfomycin may be closely associated with the mechanism of the aminoglycoside-induced nephrotoxicity, because fosfomycin did not interact directly with dibekacin.¹⁾ So far, nephrotoxicity has been ascribed, by the use of autoradiographic techniques,³⁾ to selective accumulation of aminoglycoside in the renal cortex, and, in particular, its sequestration in the lysosomes of proximal tubular cells. Further evidence was provided that aminoglycoside deposited in lysosomes inhibited the activity of lysosomal phospholipases A and C¹⁴⁾ and sphingomyelinase¹⁵⁾ which participate in the catabolism of phospho-

lipids, resulting in the impaired lysosomal function and labilization of lysosomal membrane. Morphologically, this coincided with the accumulation of phospholipid intermediates-rich myeloid bodies within lysosomes,^{4,16)} and eventual cell necrosis.

Many attempts have been made to prevent

FIG. 4. *In Vitro* Effect of Fosfomycin on Renal Lysosomal Membrane Lability induced by Dibekacin. Values represent mean $\pm$ standard deviation.

the aminoglycoside-induced nephrotoxicity by administering a protective agent. So far, L-lysine¹⁷⁾ and coenzyme Q¹⁸⁾ were found to be ineffective, but three compounds have been claimed to be protective. These were sodium D-glucarate,¹²⁾ potassium D-glucaro- δ -lactam¹³⁾ and cephalosporins, especially sodium cephalothin.¹⁹⁾ It was reported that the protective activity of sodium D-glucarate was due to the acceleration of excretion of aminoglycosides.¹²⁾ Generally, aminoglycosides including dibekacin are excreted mainly into urine. Therefore, their pharmacokinetic behavior is much influenced by the renal function. If fosfomycin had accelerating effect on the renal excretion of dibekacin in the dehydrated rats, it would be observed at low dose (0.56 mg/kg) causing no nephrotoxicity of dibekacin. In our study, however, fosfomycin had no accelerating effect on the renal excretion of dibekacin not only at low dose in the dehydrated rats but also at high dose (30 mg/kg) in rats with free access to water (Fig. 1,2). In the latter group given 30 mg/kg of dibekacin alone, no kidney injury was observed, and the renal function remained in the normal

TABLE IV. *Structure and Protective Activity of Fosfomycin Analogs in the Dehydrated Rats*

	Non-drug control	Dibekacin	Dibekacin (30 mg/kg) plus analogs (120 mg/kg)					
			Fosfomycin	(+)-Epoxypropylphosphonate	Dihydroxypropylphosphonate	cis-Propenylphosphonate	Phosphonomethylphosphonate	Phosphorylethanolamine
n	5	5	5	5	5	5	5	5
Structure
BUN (mg/dl)	26.2 $\pm$ 5.2	58.7 $\pm$ 14.9 $\uparrow$	24.0 $\pm$ 1.7	26.9 $\pm$ 2.7	34.0 $\pm$ 6.4 $\uparrow$	42.1 $\pm$ 10.4 $\uparrow$	40.2 $\pm$ 5.0 $\uparrow$	92.9 $\pm$ 32.0 $\uparrow$
Creatinine (mg/dl)	0.63 $\pm$ 0.05	0.68 $\pm$ 0.05	0.55 $\pm$ 0.05	0.60 $\pm$ 0.07	0.62 $\pm$ 0.08	0.62 $\pm$ 0.08	0.65 $\pm$ 0.05	0.78 $\pm$ 0.19
Relative kidney wt.	1.07 $\pm$ 0.08	1.16 $\pm$ 0.09	1.08 $\pm$ 0.04	1.09 $\pm$ 0.08	-----	1.14 $\pm$ 0.07	1.29 $\pm$ 0.07 $\uparrow$	1.13 $\pm$ 0.04

Results are expressed as mean $\pm$ standard deviation.

$\uparrow$ significantly different from the non-drug control ($p < 0.05$).

range (Table II).

In contrast, when dibekacin was administered to the dehydrated rats at high dose causing acute renal failure, fosfomycin showed protective effect against dibekacin-induced nephrotoxicity and consequently kept the half-life and the urinary excretion of dibekacin to the normal level (Table I, Fig. 1,2). In other words, the improvement of the delayed excretion of dibekacin at high dose in the dehydrated rats was not the direct action of fosfomycin but a secondary effect arising from the protection of kidney damage by stabilization of lysosomal membrane as follows. In view of the fact that lysosomes in the proximal tubular cells are major targets of aminoglycosides, the effect of fosfomycin on the lysosomal membrane would be critical for the protection. In fact, the protective effect of fosfomycin on lysosomal injury caused by dibekacin was shown by the reduction of myeloid bodies in the lysosomes, and by the *in vivo* and *in vitro* protection of membrane integrity of the lysosomal preparation from dibekacin. Our *in vitro* result was in agreement with the studies on other aminoglycosides.²⁰⁾ These results suggested that fosfomycin might lower the transport of aminoglycoside into the lysosomes, due to the stabilization of the membrane. In this context, it was reported that fosfomycin suppressed the release of histamine from mast cells and pulmonary tissues, which was caused by the action of aminoglycosides.²¹⁾ Our experiment indicated that fosfomycin at 100 mg/l could inhibit the degranulation of rat mast cells induced by 100 mg/l of dibekacin (data are not shown). This action of fosfomycin was correlated with an increase of cyclic AMP level, which might counteract with the labilizing effect of aminoglycosides on lysosomal membrane and release of lysosomal enzymes,²²⁾ and possibly with inhibitory action of aminoglycosides on membraneous phospholipases.¹⁴⁾

Finally, the effect of fosfomycin sodium was examined from a point of contribution from sodium cation. It is known that nephrotoxicity of aminoglycosides is promoted by feeding rats

with sodium-deficient diet,²³⁾ and that sodium sulfate protects the rats from the nephrotoxicity under certain circumstances.¹³⁾ Our study on the structure-activity relation revealed that the effect of sodium cation would be weaker than that of fosfomycin anion, because trisodium phosphonoformate was weaker in activity than fosfomycin disodium and because fosfomycin calcium given orally exhibited the protective action.¹⁾

Among six phosphonate anions, two epoxy compounds (fosfomycin and its stereoisomer) gave the best protection, suggesting the importance of an epoxy group. The stereoisomer is devoid of antibacterial activity, and hence the mechanism of protection should be different from the antibacterial mechanism. In the latter case, entry of fosfomycin into bacterial cells *via* the active transport systems of α -glycerol-3-phosphate and glucose-6-phosphate was important for the bio-activity.²⁴⁾ It would be of interest in this connection to see whether fosfomycin anion inhibits entry of aminoglycoside into the proximal tubular cells, by its competition with the anionic phosphoinositides, because phosphoinositides were reported to function as the apical membrane receptor for cationic aminoglycosides,²⁵⁾ which was essential for the entry by pinocytosis of aminoglycoside to proximal tubular cells.^{4,16)} This is a subject of future investigation.

Acknowledgement We thank Professor J.P.Fillastre, Université de Rouen for his kind suggestions and comments on this work. We are also indebted to Mr. T.Saito for supply of fosfomycin analogs.

REFERENCES

- 1) S.Inouye, T.Niizato, U.Takeda and T.Koeda: Protective effect of fosfomycin on the experimental nephrotoxicity induced by dibekacin, *J. Pharm. Dyn.*, **5**, 659–669 (1982).
- 2) I.Komiya, K.Umemura, M.Fujita, A.Kamiya, K.Okumura and R.Hori: Mechanism of renal distribution of aminoglycoside antibiotics, *J. Pharm. Dyn.*, **3**, 299–308 (1980).
- 3) F.J.Silverblatt and C.Kuehn: Autoradiography of gen-

- tamicin uptake by the rat proximal tubule cells, *Kidney Int.*, **15**, 335–345 (1979); A.Vandewalle, N.Farman, J.P.Morin, J.P.Fillastre, P.Y.Hatt and J.P.Bonvalet: Gentamicin incorporation along the nephron: autoradiographic study on isolated tubules, *Kidney Int.*, **19**, 529–539 (1981).
- 4) J.P.Morin, G.Viotte, A.Vandewalle, F.van Hoof, P.Tulkens and J.P.Fillastre: Gentamicin-induced nephrotoxicity: a cell biology approach, *Kidney Int.*, **18**, 583–590 (1980).
 - 5) O.Menlemons: Determination of total protein in spinal fluid with sulfosalicylic acid and trichloroacetic acid, *Clin. Chim. Acta*, **5**, 757–761 (1960).
 - 6) J.Borooah, D.H.Leaback and P.G.Walker: Studies on glucosaminidase. 2. Substrates for *N*-acetyl- β -glucosaminidase, *Biochem. J.*, **78**, 106–110 (1961).
 - 7) F. Wróblewski and J.S.La Due: Lactic dehydrogenase activity in blood, *Proc. Soc. Exptl. Biol. Med.*, **90**, 210–213 (1955).
 - 8) S.Momose, K.Hirata, H.Hirata, K.Goto, I.Morita, T.Takamatsu, T.Ueda, T.Iwayama, T.Ishikawa, K.Kato, H.Hirata and E.Katayama: A new approach to the pathogenesis of acute tubular necrosis: with special reference to tubular damages occurred from coincidental administration of aminoglycoside antibiotics and sodium alginate, *Nippon Hinyokikagaku Kaishi*, **60**, 823–833 (1969); K.Hirata, T.Ueda, K.Morita, H.Hirata, C.Takamatsu, K.Goto, H.Hirata, R.Aramaki, M.Kai and T.Ishikawa: Studies on pathogenesis of acute renal failure after administration of antibiotics with plasma expander, *Jap. J. Nephrol.*, **15**, 371–391 (1973).
 - 9) G.H.Hottendorf, D.Barnett, L.L.Gordon, E.F.Christensen and H.Madissoo: Nonparallel nephrotoxicity dose-response curves of aminoglycosides, *Antimicrob. Agents Chemother.*, **19**, 1024–1028 (1981); G.B.Appel and H.C.Neu: Gentamicin in 1978, *Ann. Intern. Med.*, **89**, 528–539 (1978).
 - 10) K.Furuno, K.Ando, S.Suzuki and K.Hirata: Effect of D-glucarates on basic antibiotic-induced renal damage in rats, *J. Antibiot. (Tokyo)*, **29**, 187–194 (1976).
 - 11) D.H.Lawson, W.J.Tilstone and R.F.MacAdam: Aminoglycoside-diuretic nephrotoxicity, a comparison of gentamicin and netilmicin in the rat, *Clin. Trials J.*, **17**, 338–345 (1980).
 - 12) K.Furuno, S.Matsubara, K.Ando and S.Suzuki: Preventive effect of D-glucarate against renal damage induced by kanamycin, *J. Antibiot. (Tokyo)*, **29**, 950–953 (1976); K.Furuno, S.Suzuki and K.Hirata: Influence of dehydration and D-glucarate on distribution and excretion of kanamycin in rats, *Jpn. J. Pharmacol.*, **27**, 371–378 (1977).
 - 13) T.Niizato, T.Koeda, T.Tsuruoka, S.Inouye and T.Niida: Protective effect of D-glucaro- δ -lactam against aminoglycoside-induced nephrotoxicity in rats, *J. Antibiot. (Tokyo)*, **29**, 833–840 (1976).
 - 14) K.Y.Hostetler and L.B.Hall: Inhibition of kidney lysosomal phospholipases A and C by aminoglycoside antibiotics: possible mechanism of aminoglycoside toxicity, *Proc. Natl. Acad. Sci., USA*, **79**, 1663–1667 (1982).
 - 15) J.P.Morin, G.Viotte, F.van Hoof, P.Tulkens, M.Godin and J.P.Fillastre: Functional, biochemical and morphological events related to gentamicin therapy in rats, *Drugs Exp. Clin. Res.*, **7**, 345–348 (1981).
 - 16) S.Feldman, M.-Y.Wang and G.J.Kaloyanides: Aminoglycosides induce a phospholipidosis in the renal cortex of the rat: an early manifestation of nephrotoxicity, *J. Pharmacol. Exp. Ther.*, **220**, 514–520 (1982).
 - 17) D.N.Gilbert, S.J.Kohlhepp, P.W.Kohnen, W.C.Elliott and W.M.Bennett: Failure of lysine to prevent experimental gentamicin nephrotoxicity, *J. Infectious Dis.*, **145**, 129 (1982).
 - 18) D.T.Plummer, S.Noorazar and J.D.Haslam: The effect of gentamicin and coenzyme Q on the excretion of enzymes and ions in rat urine, *Colloq.-Inst. Natl. Sante Rech. Med.*, **102**, 179–194 (1982).
 - 19) P.Dellinger, T.Murphy, V.Pinn, M.Barza and L.Weinstein: Protective effect of cephalothin against gentamicin-induced nephrotoxicity in rats, *Antimicrob. Agents Chemother.*, **9**, 172–178 (1976).
 - 20) J.P.Morin, J.P.Bendirdjian, J.P.Fillastre and J.P.Vaillant: Interference of fosfomycin with lysosomal membrane integrity of rat kidney cells, *Drugs Exptl. Clin. Res.*, **4**, 63–66 (1978).
 - 21) R.Greco, M.L.Schinitti and A.Bertelli: Phosphonomycin activity on rat mast cells, "Proc. Ist Mediterranean Congr. Chemother.," Madrid, Sept., 1978, pp. 741–746; A.Bertelli and L.Giovannini: Fosfomycin inhibition of pulmonary histamine release induced by aminoglycosides, *Drugs Exptl. Clin. Res.*, **6**, 329–332 (1980).
 - 22) M.L.Schinitti, R.Greco, L.Giovannini and A.Bertelli: *In vitro* effect of phosphomycin on intracellular cyclic nucleotides and histamine release, *Drugs Exptl. Clin. Res.*, **4**, 53–56 (1978).
 - 23) W.M.Bennett, M.N.Hartnett, D.Gilbert, D.Houghton and G.A.Porter: Effect of sodium intake on gentamicin nephrotoxicity in the rat, *Proc. Soc. Exp. Biol. Med.*, **151**, 736–738 (1976); R.D.Adelman and J.Wright: Natural Na loading. A critical variable in experimental nephrotoxicity studies in the rat, *Clin. Res.*, **30**, 153A (1982).
 - 24) F.M.Kahan, J.S.Kahan, P.J.Cassidy and H.Kropp: The mechanism of action of fosfomycin (phosphonomycin), *Ann. New York Acad. Sci.*, **235**, 364–386 (1974).
 - 25) M.Sastrasinh, T.C.Knauss, J.M.Weinberg and H.D.Humes: Identification of the gentamicin receptor of renal brush border membranes, *Kidney Int.*, **19**, 213A (1981).