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Toxicological Approaches to Streptothricin Antibiotics. III. Biological Studies on Delayed Toxicity of Streptothricin Antibiotics in Rats[1]

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To delineate the time of onset and the mechanism of delayed toxic effects produced by streptothricin antibiotics in rats, the tissue antibiotic distribution, histopathologic features of the kidney, serum biochemical changes and antibiotic metabolites recovered in the urine after administration of a radiolabeled compound were investigated. Rats showed a pattern of tissue antibiotic distribution similar to that observed in the mouse. Microscopic examination of the kidney revealed lesions which became evident at 48 hr after the injection. Serum biochemical tests showed a rapid elevation of blood urea nitrogen and creatinine nitrogen levels from 48 hr post injection, with abnormalities in the cellulose acetate membrane electrophoresis pattern of serum proteins. Thus, nephrotoxic effects evoked by the antibiotic became evident about 48 hr after the injection, indicating that the time of onset of delayed toxicity of the compound is approximately 48 hr after dosing. As for the mechanism of the delayed toxic effects, evidence has been obtained for the formation of a toxic metabolite with an opened lactam ring, or an "acid compound" excreted in the rat urine. These findings support our previous inference, based on studies in mice.

Keywords——racemomycin-D; "opened lactam" substance; onset of delayed nephrotoxic effect; ^{14}C-glycyl-racemomycin-A; renal damage; serum biochemical test; the tissue antibiotic distribution

Previous studies in this laboratory on the mechanism of delayed toxicity of streptothricin antibiotics dealt with the tissue distribution in mice[3] and histopathologic observations in rats,[4] and indicated a marked nephrotoxic potential. The authors suggested that an "opened lactam" substance detected as a metabolite of the antibiotic in urine might be responsible for the delayed toxicity.[4] However, the previous study dealt only with the histopathologic findings 96 hr after administration, and did not provide any information on the time of onset of delayed toxic effects.[4] Thus, the present study was undertaken to investigate the time of onset and the mechanism of delayed nephrotoxicity of streptothricin antibiotics in rats from a comparartive toxicological standpoint by assessment of the tissue antibiotic distribution, by histopathologic examination of the kidney, and by serum biochemical tests at various times after administration.

Materials and Methods

Animals——Male rats of the Wistar strain weighing 200 to 220 g were used.
Antibiotics——Racemomycin-D[5] and ^{14}C-labeled glycyl-racemomycin-A,[4] were used.

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3) Y. Inamori, S. Sunagawa, M. Tsuruga, Y. Sawada, H. Taniyama, G. Saito and K. Daigo, *Chem. Pharm. Bull.* (Tokyo), **26**, 1147 (1978).
4) Y. Inamori, K. Morimoto, K. Morisaka, G. Saito, Y. Sawada, H. Taniyama and H. Matsuda, *Chem. Pharm. Bull.* (Tokyo), **27**, 237 (1979).
5) Y. Inamori, S. Sunagawa, M. Tsuruga, Y. Sawada and H. Taniyama, *J. Ferment. Technol.*, **56**, 15 (1978).

Administration and Dose——Both antibiotics were made up as solutions in distilled water for injection and administered intravenously *via* the caudal vein at the doses described elsewhere.[3,4]

Blood Level, Distribution in Organs and Urinary Recovery of Antimicrobial Activity Following Administration of Racemomycin-D, and Histopathologic Examination of the Kidney——The methods employed were essentially the same as those described in the preceding reports.[3,4]

Serum Biochemical Tests after Administration of Racemomycin-D——Blood samples drawn from rats at selected intervals after drug administration were assayed for various serum biochemical parameters with an autoanalyzer, using Smac system.

Cellulose Acetate Membrane Electrophoresis of Serum and Urine Following Administration of Racemomycin-D——Individual serum samples or solutions of lyophilized urine specimens were applied to cellulose acetate membrane in quantities of 1 μl per cm. Electrophoretic separation was carried out (1 cm, 0.6 mA, 40 min) and the membranes were stained with ponceau 3R.

Metabolites in Rat Urine after the Administration of ^{14}C-Labeled Glycyl-racemomycin-A——Rats were dosed with ^{14}C-labeled glycyl-racemomycin-A (338168 dpm/rat) and maintained in urine collection cages to obtain 24 hr urine specimens. These were examined as described elsewhere.[4]

Antimicrobial Activity Assay——The assay was carried out by the paper disc method as previously reported,[3] using *Bacillus subtilis* PCI-219 (10^6 cells per ml) as the test organism, and the antibacterial activity was calculated from calibration curves.

Results

Antimicrobial Activity Distribution in Rat Tissues after the Administration of Racemomycin-D

Blood Antibiotic Level——Blood antibiotic levels in rats after the administration of racemomycin-D were determined by serum assays for antimicrobial activity. As shown in Fig. 1, there was a rapid dissipation of activity from the blood after injection, as observed previously in the mouse.[3]

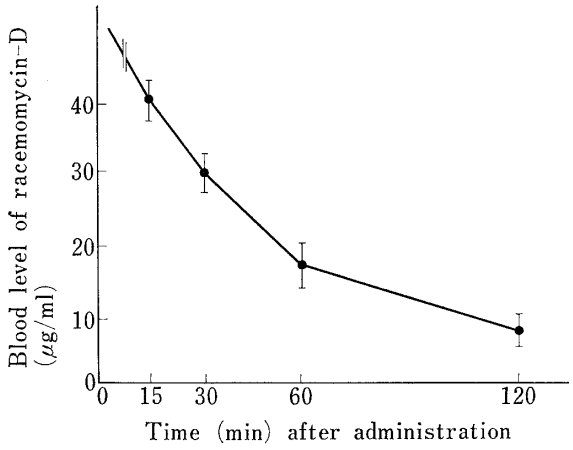


Fig. 1. Apparent Blood Level of Racemomycin-D as Antimicrobial Activity in Rats after Intravenous Administration

Mean±S.E. (n=4).

Table I. Urinary Recovery of Antimicrobial Activity after Administration of Racemomycin-D to Rats

Time (hr)	Concentration (μg/ml)	Recovery (%)
0—24	54.50	19.82
24—48	7.55	2.75
48—72	3.60	1.31

Rats were dosed intravenously with 40 mg/kg of racemomycin-D in water.
Recovery in the urine was determined during 0—24, 24—48 and 48—72 hr after the injection.

Urinary Recovery of Antimicrobial Activity——The urinary recovery of the antibiotic was assessed in rats following single intravenous doses. The data obtained showed that most of the total antimicrobial activity excreted in the urine was recovered within 24 hr after injection (Table I). This is supported by the finding that rats dosed with ^{14}C-labeled glycyl-racemomycin-A showed remarkably low values for urinary recovery from 24 hr post injection onwards (data not presented here). The results indicate, moreover, that a considerable proportion of racemomycin-D administered is excreted as inactive metabolites in the urine.

Antibiotic Distribution in Organs——Table II shows the levels of antimicrobial activity in various organs of the rat after intravenous injection of racemomycin-D. Racemomycin-D

Table II. Distribution of Racemomycin-D in Rat Organs after *i.v.* Administration

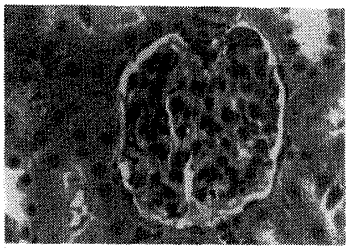
Organ	Time (hr) after administration (40 mg/kg)					
	1	3	5	24	48	72
Brain	—	—	—	—	—	—
Lung	4.60	±	—	—	—	—
Heart	0.84	—	—	—	—	—
Liver	—	—	—	—	—	—
Adrenal gland	—	—	—	—	—	—
Spleen	0.98	±	—	—	—	—
Kidney	217.78	104.00	136.00	192.00	164.00	84.43
Bladder	24.40	2.36	1.14	±	—	—
Testicle	—	—	—	—	—	—
Stomach	—	—	—	—	—	—
Small intestine	—	—	—	—	—	—
Large intestine	—	—	—	—	—	—

Values are the means of 6 animals (μg/g).
—, not detected; +, detected.

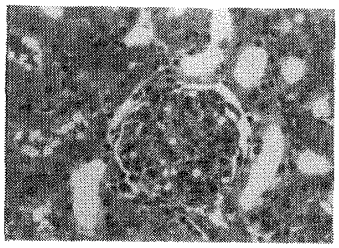
was found to be distributed at higher levels for a longer period in the kidneys than in any other organ. The tissue antibiotic distribution pattern is very similar to that observed in the mouse, though the actual levels differed.

Histopathologic Findings in the Kidneys of Rats Receiving Racemomycin-D

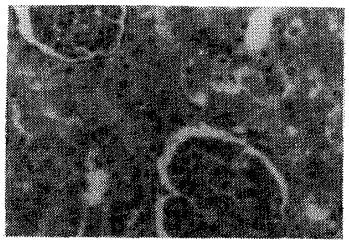
Renal tissues obtained from rats at various times after intravenous administration of racemomycin-D were examined by light microscopy. Photomicrographs of representative sections are presented in Figs. 2a to 2e. At 24 hr after injection, there was no appreciable



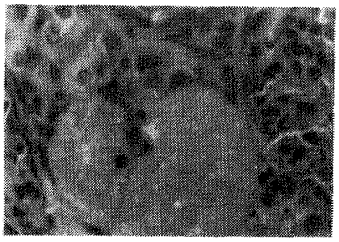
a control (normal rat)



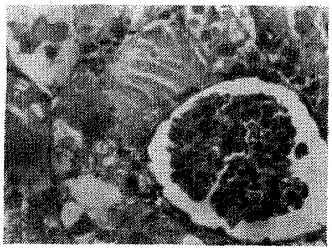
b 24 hr after injection



c 48 hr after injection



d 72 hr after injection



e 96 hr after injection

Fig. 2a—e. Photographs of the Renal Cortex of Normal Rats and Rats Administered Racemomycin-D (Hematoxylin-Eosin Staining)

histopathologic changes in the kidneys of treated rats (Fig. 2b) as compared with the control group (Fig. 2a). From about 48 hr after the injection karyolysis and necrosis of the renal tubular epithelium became evident (Fig. 2c), and tissues at 72 hr showed calcification in addition to hyaline casts and tubular necrosis (Fig. 2d). At 96 hr, there was marked cortical atrophy with necrosis and hemorrhage; the proximal renal tubular epithelium of the cortex was largely degenerated and necrotized, with calcification in the necrotic areas (Fig. 2e). However, the basement membrane of the uriniferous tubules remained and there were sporadic areas of regeneration of the distal tubular epithelium characterized by mitotic figures in the cortex. Furthermore, the proximal tubular epithelium at the cortico-medullary junction showed cloudy swelling, and numerous hyaline casts were seen in the lumen of the loop of Henle in the inner zone of the medulla. In contrast, little or no glomerular abnormality was seen in the histopathologic examination of the renal tissues at any period after the injection, with the exception of dilatation of Bowman's space noted at 96 hr.

Serum Biochemical Findings in Rats after Intravenous Injection of Racemomycin-D

An attempt was made to estimate the time of onset of delayed toxicologic effects in rats from the values obtained in the serum biochemical tests performed at intervals after a single *i.v.* dose of racemomycin-D. The results are summarized in Table III. Blood urea nitrogen (BUN) and creatinine nitrogen (CreN) levels, known to be parameters of renal impairment, showed no changes during the first 24 hr after the injection, compared to the control group. Both parameters, however, showed a sharp increase from 48 hr onwards, reacting levels about ten (BUN) and seven (CreN) times the normal values at 72 hr, at which time deaths began to occur. A decline in serum iron concentration, which is frequently associated with renal damage, was also noted and became increasingly pronounced with time after the injection.

Table III. Clinico-Chemical Data for Rats after Racemomycin-D Administration

		Control	1	3	5	24	48	72 (hr)
T P	g/dl	6.18± 0.39	6.12± 0.25	5.77± 0.27	5.88± 0.43	6.70± 0.12	6.85± 0.76	6.33± 0.54
BUN	mg/dl	21.60± 4.30	14.87± 3.13	15.40± 2.87	15.00± 4.15	26.67± 3.68	115.50± 13.3	231.33± 51.5
CreN	mg/dl	0.83± 0.76	0.38± 0.09	0.63± 0.09	0.62± 0.10	1.13± 0.13	3.97± 0.96	6.13± 0.83
A L B	g/dl	2.48± 0.16	2.83± 0.22	2.43± 0.04	2.66± 0.31	2.90± 0.07	3.20± 0.67	3.17± 0.54
T Chol.	mg/dl	69.80± 8.38	71.67± 26.8	66.50± 5.91	68.40± 9.83	96.50± 10.5	109.00± 13.7	100.33± 9.88
T G	mg/dl	39.25± 10.2	25.66± 10.4	32.00± 16.0	34.25± 12.9	25.50± 10.6	61.17± 12.0	63.50± 7.50
Fe	μg/dl	180.25± 28.3	102.80± 15.4	95.20± 20.4	100.00± 26.0	71.25± 25.5	47.17± 10.9	38.50± 10.5
Na	mEq/L	142.80± 3.06	148.33± 4.92	145.00± 2.94	141.40± 4.88	137.75± 2.86	140.33± 1.89	140.33± 2.62
Cl	mEq/L	107.60± 2.81	104.67± 2.81	103.33± 2.43	104.60± 3.83	101.00± 0.71	105.33± 4.03	96.67± 0.47
A/G		0.60± 0.00	0.85± 0.19	0.73± 0.04	0.82± 0.07	0.78± 0.04	0.78± 0.04	1.06± 0.27

Each value represents the mean ± S.E. of 6 rats.
Racemomycin-D, 40 mg/kg.

The animals also exhibited elevation of total cholesterol and triphosphoglyceride. These observations are consistent with the observation of Shimamune *et al.* in rats with experimental renal dysfunction.[6]

6) K. Shimamune, *Rinsho-Kensa*, **20**, 185 (1975).

Cellulose Acetate Membrane Electrophoresis of Rat Serum after the Administration of Racemomycin-D

Sera from rats dosed *i.v.* with racemomycin-D were analyzed by electrophoresis employing cellulose acetate membranes to estimate the time of onset of delayed nephrotoxic effects. Figure 3 shows the results. There was no significant difference in the electrophoretic pattern of serum proteins during the first 24 hr period after administration of the antibiotic between the treated group and the control group. From 48 hr after the injection, however, α_2-globulin bands appeared, with an increase of β-globulin. The cellulose acetate membrane electrophoretic pattern of serum proteins bore a markaed resemblance to that observed in cases of renal damage.

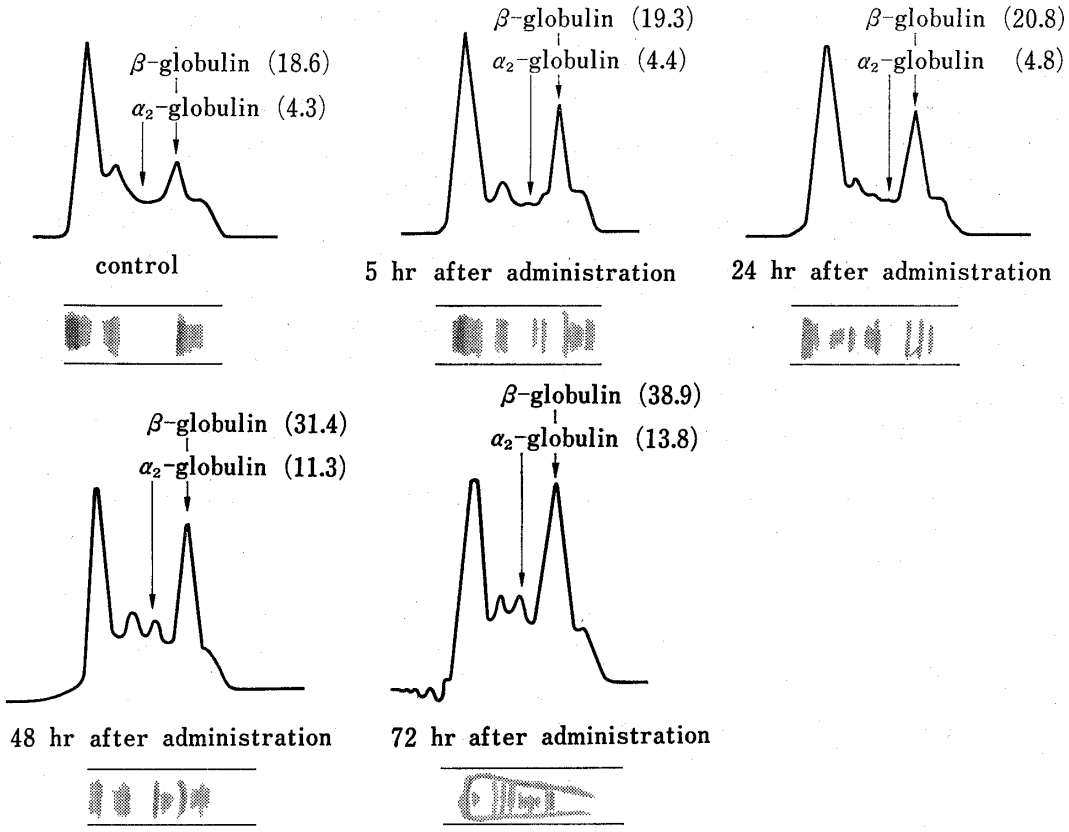


Fig. 3. Cellulose Acetate Electrophoretic Patterns of Serum Proteins in Ratsafter Racemomycin-D Administration

Urine Output and Cellulose Acetate Membrane Electrophoresis of Urinary Protein of Rats after the Administration of Racemomycin-D

The urine volume and cellulose acetate membrane electrophoretic pattern of urinary protein of rats housed in urine collection cages were determined during consecutive 24 hr periods after an intravenous injection of racemomycin-D; the results are shown in Table IV. From 48 hr post injection, the animals dosed with the antibiotic gave a reduced urine output with an altered electrophoretic pattern of urinary protein characterized by the emergence of α_2-globulin bands and an increase of β-globulin compared with the control group. Urinalysis also gave findings indicative of the onset of nephrotoxic effects at 48 hr after the injection.

Metabolites in Rat Urine after the Administration of ^{14}C-Labeled Glycyl-racemomycin-A

Urine collected from rats in urine collection cages following an *i.v.* injection of ^{14}C-glycyl-racemomycin-A was analyzed by paper chromatography for radioassay of metabolites under the conditions shown in Fig. 4. A prominent peak of radioactivity was observed at the

TABLE IV. Volumes and Cellulose Acetate Electrophoretic Patterns of Urine in Rats after Racemomycin-D Administration

	Control	Time (hr) 0–24	24–48	48–72
Volume (ml)	8.35±0.49	5.9±2.76	2.03±0.70	0.5[a]
Cellulose alb.	±	±	++	
Acetate α_1-glo	±	±	+	
Membrane α_2-glo	±	±	+++	
Electro- β-glo	−	−	+	
phoretic γ-glo	−	−	±	
Pattern				

Each value represents the mean ±S.E. of 4 rats.
a) Three rats had died by this time.
Dose, 40 mg/kg; route, intravenous administration.

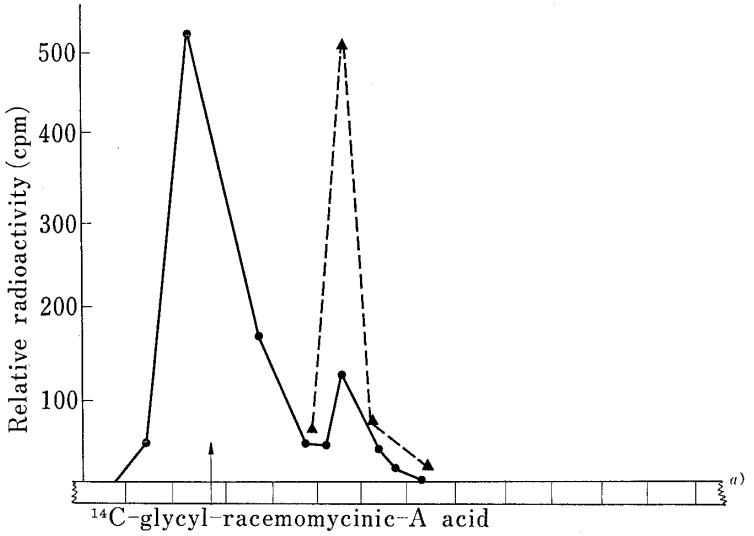


Fig. 5. Radioactivity Scanning of a Paper Chromatogram of Urine obtained after Administration of ^{14}C-Glycyl-racemomycin-A

●—●, urinary components; ▲---▲, ^{14}C-glycyl-racemomycin-A.
a) Toyo Roshi No. 51 paper; solvent, BuOH–pyridine–AcOH–H_2O–*tert*-BuOH (15:10:3:12:4).

Rf corresponding to "opened lactam" substance, in accord with previously reported findings in the mouse.[4]

Discussion

Rats dosed with streptothricin antibiotic showed a tissue distribution of antimicrobial activity very similar to that observed in mice. Namely, the antibiotic dissipated rapidly from the circulation after the injection (Fig. 1), indicating rapid excretion and distribution into organs of the streptothricin antibiotic. Evidence has been obtained for the distribution of the antibiotic to the kidneys at higher concentrations and for a longer period than in any other organ examined (Table II). This suggests a profound affinity of this streptothricin antibiotic for the kidney, as is the case with kanamycin.[7]

7) I. Komiya, M. Fujita, S. Murata and K. Umemura, the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1977, IV-166.

Microscopic examination of renal tissues from rats dosed with these compounds revealed lesions such as pronounced degeneration and necrosis of the proximal and distal renal tubular epithelium 48 hr after the injection (Fig. 2c). However, there were no significant changes in the renal glomeruli 96 hr after the administration. These findings suggest that the nephrotoxicity of the streptothricin antibiotics involves a mechanism resembling not so much the mechanism of puromycin[8] or viomycin[9] as that of kanamycin.[10] Serum biochemical tests showed that BUN and creatinine nitrogen levels (indicators of renal damage) remained virtually unaltered during the first 24 hr after the injection, but both showed a sharp increase from about 48 hr onwards, compared with the control group (Table III). These elevations of BUN and CreN were greater than those following a single *i.v.* dose of a similar basic antibiotic, kanamycin[11] or puromycin.[11] This suggests that streptothricin antibiotics have a profound nephrotoxic potential. Electrophoretic patterns of serum and urinary proteins on cellulose acetate membranes showed the emergence of α_2-globulin, together with an increase of β-globulin from about 48 hr onwards (Fig. 3, Table IV). The pattern is very similar to that usually observed in renal damage. In studies of consecutive 24 hr urine outputs, the urine volume showed a rapid decrease from about 48 hr on (Table IV).

From these data, the onset of the delayed nephrotoxic effect was estimated to ocuur at about 48 hr after administration.

As for the mechanism underlying the delayed toxicity, the formation of an "opened lactam" substance[12] has been demonstrated by analysis of metabolites recovered in the 24 hr urine following administration of ^{14}C-glycyl-racemomycin-A to rats, and also to mice. It also appears that a considerable proportion of streptothricin antibiotics is excreted in inactive forms in the urine. The present results support our previous inference[4] that the delayed toxicity of the antibiotic can be ascribed to the formation of a toxic metabolite, the "opened lactam" substance.

Acknowledgement We wish to express our thanks to Professor Tsunematsu Takemoto, Director of the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, for his guidance.

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11) S. Nagase and S. Tanaka, "*Jitsuken Dobutsu to Rinsho-Seikagaku no Data*," Soft Science Co., Ltd., Tokyo, 1976, p. 298.
12) H. Taniyama, Y. Sawada and T. Kitagawa, *J. Antibiot.*, **24**, 622 (1971).