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# The Mechanisms of Delayed Insecticidal Action of Streptothricin Antibiotics. II.[1] Effect of Racemomycin-D on Excretion Function of the 5th Instar Larvae of Silkworm, *Bombyx mori* LINNE

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Previously, in order to clarify the mechanisms of the insecticidal action of streptothricin antibiotics, we had investigated the distribution of racemomycin-D into the tissues of the 5th instar larvae of silkworm, *Bombyx mori,* and reported that racemomycin-D accumulated in larger quantities for a longer time in the Malpighian tubules than in any other tissue. In this work, in order to clarify the mechanism of the insecticidal action in more detail, we investigated the Malpighian tubules of the larvae histopathologically and confirmed the occurrence of severe delayed damage. It is noteworthy that streptothricin antibiotics show strong nephrotoxicity in mammals.

At 24 h after injection, which was the onset time of the damage to the Malpighian tubules, both the blood level and excretion amount of uric acid were increased, and they subsequently increased much further as the damage became more severe. Thus, through histopathologic examination and analysis of uric acid levels, we showed that the onset time of delayed toxicity of racemomycin-D in the organs of the insects was at 24 h after injection.

**Keywords**——racemomycin-D; histopathologic examination; uric acid in blood and feces; Malpighian tubules; silkworm the 5 th instar larvae, *Bombyx mori*; delayed toxicity onset time; mechanism of insecticidal action

We have shown that racemomycin-D, one of the streptothricin antibiotics, has broad-spectrum insecticidal activity.[2,3] Then, in order to clarify the mechanisms of insecticidal action of racemomycin-D, we investigated the distribution of racemomycin-D into the tissues of the 5th instar larvae of silkworm, *Bombyx mori,* and reported that racemomycin-D accumulated in larger quantities for a longer time in the Malpighian tubules than in any other tissue.[4]

In this report, we investigated the Malpighian tubule of the larvae histopathologically and examined changes of uric acid concentrations in the blood and feces.

## Materials and Methods

**Antibiotic**——Racemomycin-D[5] is one of the streptothricin antibiotics produced by *Streptomyces lavendulae* OP-2.[6]

**Insect**——The 5th instar larvae of silkworm, *Bombyx mori* L.(body weight, 2.5—3.1 g), were used.

**Administration and Dose**——Administration was done by injection as described in the previous paper.[4] The dose used was 150 $\mu$g/g, which had also been used for studies on the distribution of racemomycin-D into the tissues of the larvae in the previous paper.[4]

**Histopathological Samples of the Malpighian Tubules**——After injection of racemomycin-D, the larvae were dissected to excise the Malpighian tubule and digestive canal (from the posterior portion of ventriculus to near the rectum). Then the excised digestive canal was washed with 0.85% NaCl solution and fixed in Bouin's solution (saturated picric acid solution 75 ml, formalin 25 ml and glacial acetic acid 5 ml) for 3 h. Fixed digestive canal and Malpighian tubules were separated into the anterior portion (near the ventriculus) and the posterior portion (near the colon and rectum), and paraffin sections 5—6 $\mu$m thick were made. They were stained with hematoxylin–eosin solution and examined through an optical microscope with a magnification of 100.

**Quantitative Analysis of Blood Level of Uric Acid**——After injection of racemomycin-D, the abdominal legs of the larvae were cut off at the prescribed time and the blood was collected. Collected blood was centrifuged at 10000 rpm for 3 min, and the blood level of uric acid in the resultant serum (50 $\mu$l) was calculated using Determiner UA[7] (uricase-peroxidase method; Kyowa Hakko Co., Ltd.) with absorbance measurement at 550 nm.

**Quantitative Analysis of Uric Acid in Feces**——Feces of the larvae were collected every 24 h after injection and dried. A weighed amount of dried feces was reduced to powder (it should be noted that from 48 h after injection, fecal excretion was small because of the strong delayed toxicity of racemomycin-D), and extracted with dist.$H_2O$ in a boiling water bath for 3 h. The extract was centrifuged at 10000 rpm for 3 min and 50 $\mu$l of the extract was assayed for uric acid using Determiner UA. The uric acid content of the feces was then calculated.

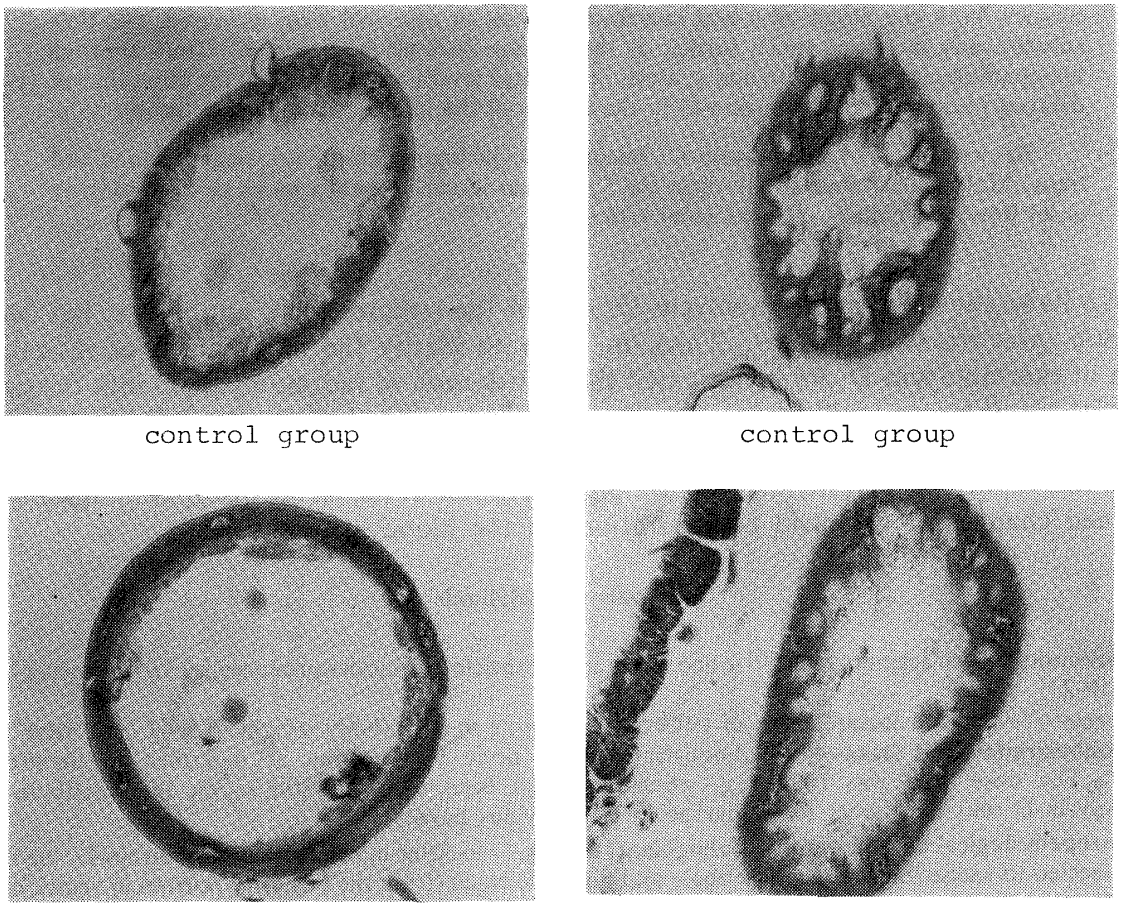
## Results

### Histopathologic Findings in the Malpighian Tubules of Larvae Given Racemomycin-D

The Malpighian tubules of the larvae were examined histopathologically at various times after injection of racemomycin-D. The results are shown in Figs. 1—4.

As shown in Fig. 1, the Malpighian tubules of the administered group had no abnormality at 12 h after injection compared with the control group.

As shown in Fig. 2, at 24 h the anterior portion of the Malpighian tubule had some coagulations of chromatin (arrow a), which is a symptom of pycnosis, but in other respects there was no abnormality compared with the control group. However, at the posterior portion the coagulated chromatin was localized in the peripheral parts of the nucleus (arrow b),



control group　　control group

treated group　　treated group

anterior portion　　posterior portion

Fig. 1. Photographs of the Malpighian Tubules of the 5th Instar Larvae of Silkworm, *B. mori*, at 12 h after Administration of Racemomycin-D Hematoxylin-Eosin Staining ×100

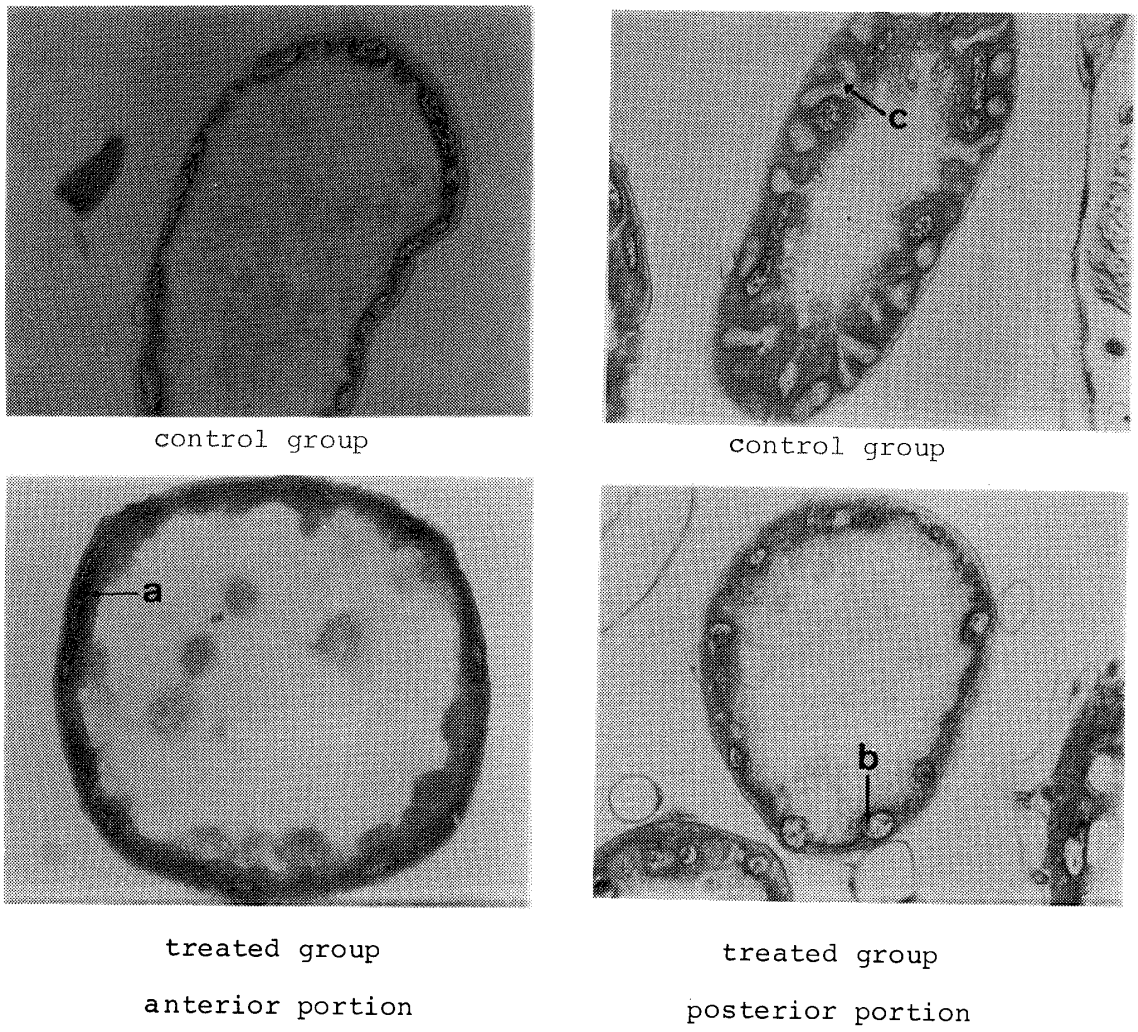



Fig. 2. Photographs of the Malpighian Tubules of the 5th Instar Larvae of Silkworm, *B. mori, at* 24 h after Administration of Racemomycin-D Hematoxylin-Eosin Staining ×100

a, chromatin; b, localization of chromatin; c, invagination.

and the area of cytoplasm was smaller. Further, the deep invaginations of the apiral cell membrane (arrow c), characteristic of the posterior portion, had lost their clarity.

As shown in Fig. 3, at 48 h the photograph of the anterior portion of the Malpighian tubule was similar to that at 24 h, but in the lumen of the tubule many secretions could be found. However, at the posterior portion, cells in the lumen showed severe necrosis, and only the basement membrane was still alive (arrow a). In the lumen of the posterior tubule, there were many disintegrated fragments of cytoplasm (arrow b) and scattered chromatin substances (arrow c), both resulting from severe necrosis of the tubule cells.

At 72 h, as shown in Fig. 4, the anterior portion of the Malpighian tubule showed coagulations of chromatin which were stronger than at 48 h, and pycnosis was apparent (arrow a). The lumen of the tubule contains large amounts of disintegrated chromatin (arrow b). In the surface of the lumen of the tubule, the cell membrane was detached (arrow c) and its boundary was partially obscured. However, at the posterior portion the necrosis was as severe as at 48 h, and in the lumen of the tubule many crystals, which might be calcium oxalate, were seen (arrow d). The cytoplasm which was found at 48 h (Fig. 3, arrow b) was no longer being released and only the apparent coagulation of chromatin was detected (arrow e).

**Blood Level of Uric Acid of the Larvae after Injection of Racemomycin-D**

The blood level of uric acid in larvae was investigated after injection of racemomycin-

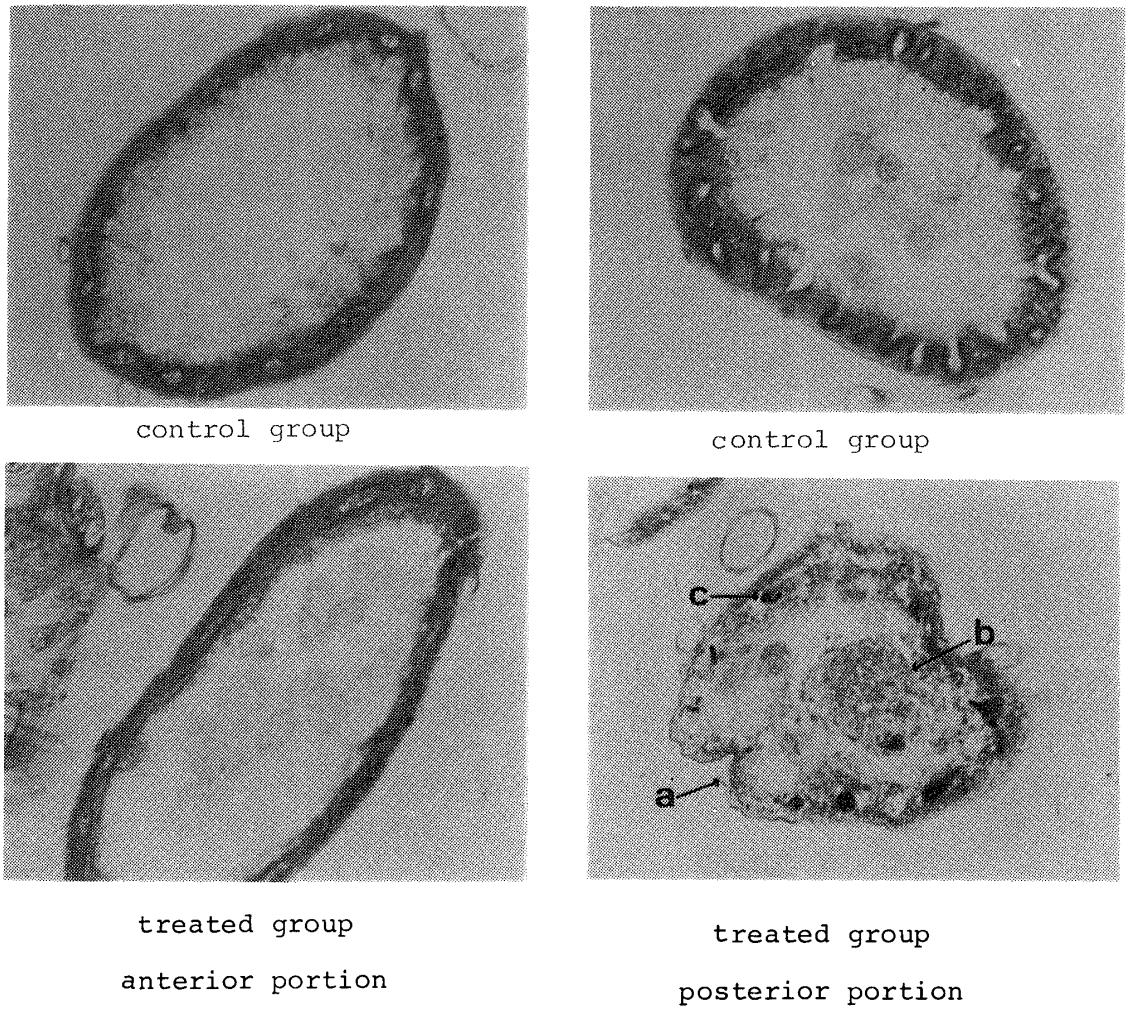



Fig. 3. Photographs of the Malpighian Tubules of the 5th Instar Larvae of Silkworm, *B. mori*, at 48 h after Administration of Racemomycin-D Hematoxylin-Eosin Staining ×100

a, basement membrane; b, cytoplasm; c, scattered chromatin.

D. The results are shown in Table I. From 24 h the level in the administered group began to rise steeply and at 72 h it was five times that of the control group. The level of the control group at each time coincided with the value reported by Hayashi *et al.*[8]

**Concentration of Uric Acid in Feces of the Larvae after Injection of Racemomycin-D**

The quantities of uric acid excreted in feces were investigated at various times after injection of racemomycin-D. The results are shown in Table II. Up to about 12 h after injection the difference between the administered group and the control group was not large. However, from 24 h the value rose steeply and at 72 h it was ten times that of the control group. The value of the control group at each time was similar to that reported by Hashida *et al.*[9]

## Discussion

Through the histopathologic examination of the 5th instar larvae of silkworm, *Bombyx mori*, it was clarified that the insecticidal action of racemomycin-D is a result of severe damage to the Malpighian tubules (Figs. 1—4). The posterior portion of the tubule was damaged more severely than the anterior portion. The function of the posterior or cryptonephric portion of the insect Malpighian tubule is to reabsorb water from the excreta,[10] and therefore this organ

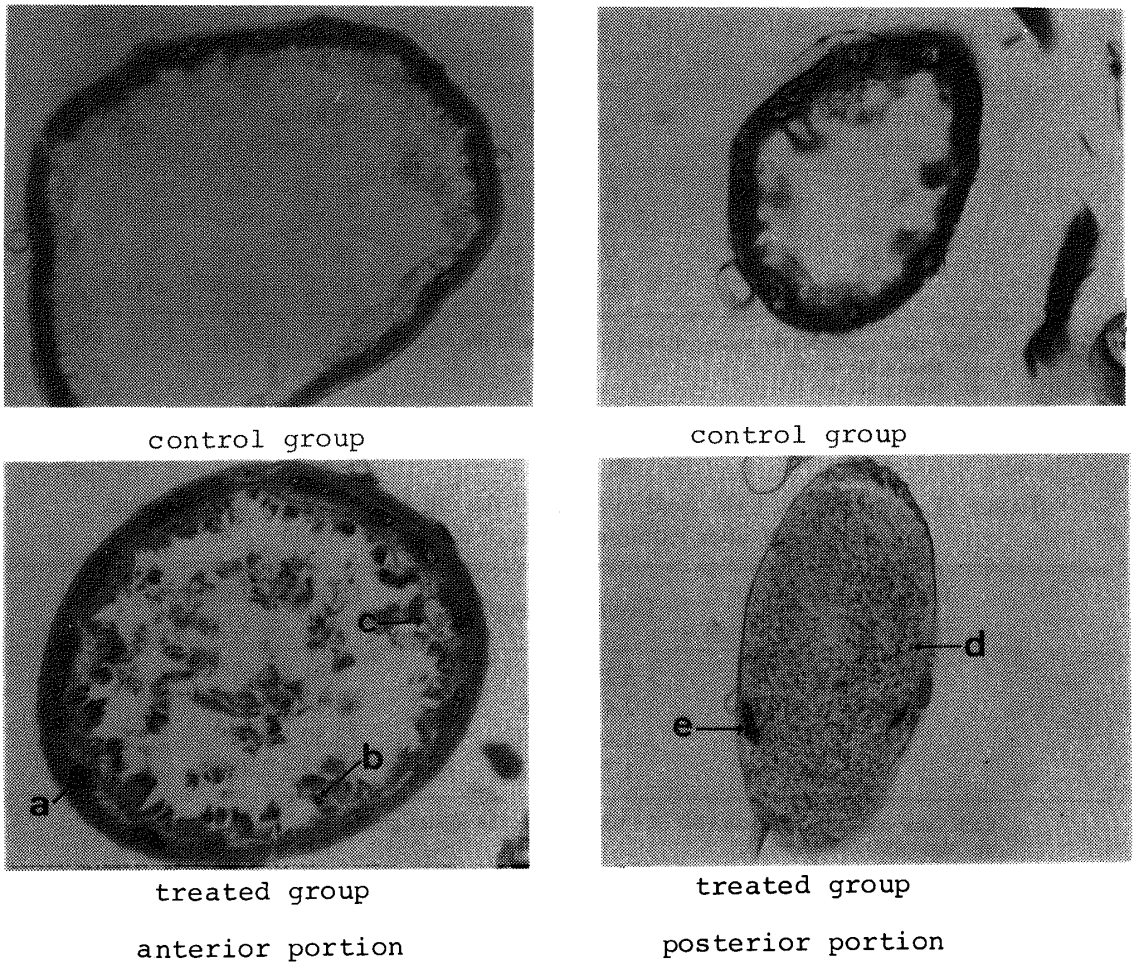



Fig. 4. Photographs of the Malpighian Tubules of the 5th Instar Larvae of Silkworm, B. *mori*, at 72 h after Administration of Racemomycin-D Hematoxylin-Eosin Staining ×100

a, pycnosis; b, released chromatin; c, detachment of cell membrane; d, crystals possibly calcium oxalate; e, released chromatin.

may be comparable to the proximal renal tubule in the mammalian kidney in this respect. It has already been reported that streptothricin antibiotics showed strong delayed nephrotoxicity in mammals,[11] and we have recently reported that streptothricin antibiotics damage the proximal renal tubule severely.[12,13] It is noteworthy that the antibiotic acts against much the same excretory organs in both mammals and insects, and in both cases causes delayed damage.

In the case of phytophagous insects like the silkworm, the cells of the digestive canal carry out active transport of certain cations,[14] and thus have some functional resemblance to the cells of the Malpighian tubule. Nevertheless, it is noteworthy that in the present histopathologic observations, only the Malpighian tubule cells were found to be severely damaged while the digestive canal was less affected. This is interesting from a comparative physiological viewpoint, since it means that the selective toxic effect of racemomycin-D is focused exclusively on the excretory organs such as insect Malpighian tubule and mammalian kidney. This is consistent with the previous report that racemomycin-D accumulated at higher levels for a longer time in the Malpighian tubules than in any other tissue of the 5th instar larvae of silkworm, and that the Malpighian tubules changed color. We have already reported that in the case of mammals 1) streptothricin antibiotics show a strong delayed toxicity,[5] 2) they accumulate in higher levels for a longer time than in the kidney,[12,13,15] 3) a strong nephrotoxicity[12,13] was found through histopathologic examination. It is noteworthy that in insects and mammals (invertebrates and vertebrates), racemomycin-D accumulates in the excretory organs

TABLE I. The Blood Level of Uric Acid of the 5th Instar Larvae of Silkworm, *B. mori*, after Injection of Racemomycin-D

| Time (h) after administration | Control group | Treated group (150 μg/g) |
|---|---|---|
| 12 | 6.10±1.04 | 10.17±3.14[a] |
| 24 | 7.53±2.78 | 26.72±4.08[a] |
| 48 | 9.97±1.29 | 26.74±4.28[a] |
| 72 | 10.55±1.77 | 49.27±11.86[b] |

Each value represents the mean±SD of 3 silkworms in the control group.
*a*) Mean±SD ($n$=6).
*b*) Mean±SD ($n$=3).
Assay method: Uricase-peroxidase method.
Unit: mg/ml.

TABLE II. Amount of Uric Acid in Feces of the 5th Instar Larvae of Silkworm, *B. mori*, after Administration of Racemomycin-D

| Time (h) after administration | Control group | Treated group (150 μg/g) |
|---|---|---|
| 12 | 3.28±0.61 | 4.91±0.67[a] |
| 24 | 2.41±0.16 | 8.34±0.19[a] |
| 48 | 1.46±0.05 | 13.50±0.37[a] |
| 72 | 1.34±0.03 | 14.22±2.95[b] |

Each value represents the mean±SD of 5 silkworms in the control group.
*a*) Mean±SD ($n$=6).
*b*) Mean±SD ($n$=3).
Assay method: Uricase-peroxidase method.
Unit: mg/g.

in large quantities for a long time, and only these organs are damaged on the basis of histopathologic observations.

The relationship between delayed toxicity of racemomycin-D and changes of concentration of uric acid in the larvae were investigated. Uric acid in the insects, like urea in mammals, is the main nitrogen compound excreted (80% of the nitrogen excreted is said to be uric acid). Hence, it was of interest to examine the changes of concentration of uric acid. From 24 h after injection, the values of uric acid in the blood and feces of the administered group began to increase steeply compared with the control group (Tables I and II). Thus, histopathologic examination (Figs. 1—4) confirmed a relationship between the concentration of uric acid and the delayed toxicity at 24 h after injection of racemomycin-D when damage to the Malpighian tubules first became apparent.

In the case of rats, renal damage began to be seen histopathologically at 48 h after injection of racemomycin-D, when the BUN (blood urea nitrogen) level began to increase steeply.[13] However, it is still not clear whether the increase of uric acid is caused by the decline of the excretion function on account of the damage to the Malpighian tubules or whether it results from abnormal metabolism induced by racemomycin-D. Further studies, including the measurement of xanthine dehydrogenase activity and others, are in progress.

Finally, the onset time of the delayed toxicity of racemomycin-D in the larvae was estimated to be approx. 24 h after injection for the following reasons.

1) Slight damage to the Malpighian tubules was first detected at 24 h through histopathologic examination.

2) The values of uric acid in the blood and feces also increased steeply at 24 h.

The results are consistent with the following findings in the investigation of distribution of antibiotic into the tissues of larvae given racemomycin-D. 1) From 24 h after injection, the

body weight decreased remarkably. 2) The amounts ingested became less and spontaneous movement decreased. 3) The amounts of feces decreased remarkably. At 48 h after injection, deaths were found among the larvae. These results are comparable with the data on mammals, already reported by the authors. That is, in the case of the mammals the onset time of the delayed toxicity in the kidneys is at 48 h after injection, and deaths occur from 72 h.[13]

In conclusion, it is clear that the insecticidal action of streptothricin antibiotics is due to delayed damage to the Malpighian tubules, and that the onset time of the delayed toxicity in this organ is at 24 h after injection.

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## References and Notes

1) This work was presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April, 1982.
2) T. Takemoto, Y. Inamori, Y. Kato, M. Kubo, K. Morimoto, K. Morisaka, M. Sakai, Y. Sawada and H. Taniyama, *Chem. Pharm. Bull.*, **28**, 2884 (1980).
3) M. Kubo, Y. Kato, K. Morisaka, Y. Inamori, K. Nomoto, T. Takemoto, M. Sakai, Y. Sawada and H. Taniyama, *Chem. Pharm. Bull.*, **29**, 3727 (1981).
4) The distribution of racemomycin-D into the tissues of the 5th instar larvae of silkworm, *Bombyx mori*, was reported at the 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April, 1981.
5) Y. Inamori, S. Sunagawa, M. Tsuruga, Y. Sawada and H. Taniyama, *J. Ferment. Technol.*, **56**, 15 (1978).
6) Y. Inamori, S. Sunagawa, Y. Sawada and H. Taniyama, *Hakko-Kogaku-Kaishi*, **54**, 795 (1976).
7) N. Kageyama, *Clin. Chim. Acta.*, **31**, 421 (1971).
8) Y. Hayashi, *J. Sericult. Sci. Japan*, **30**, 89 (1961).
9) A. Hishida and Y. Hayashi, *J. Sericult. Sci. Japan*, **31**, 69 (1962).
10) V. B. Wigglesworth, "Principles of Insect Physiology," 7th ed., John Wiley & Sons, New York, 1972.
11) G. Rake, D. Hamre, F. Kavanagh, W. Koerber and R. Donovick, *Am. J. M. Sc.*, **210**, 61 (1945).
12) Y. Inamori, K. Morimoto, K. Morisaka, G. Saito, Y. Sawada and H. Taniyama, *Chem. Pharm. Bull.*, **27**, 230 (1979).
13) Y. Inamori, Y. Kato, K. Morimoto, K. Morisaka, G. Saito, Y. Sawada and H. Taniyama, *Chem. Pharm. Bull.*, **27**, 2570 (1979).
14) E. Anderson and W.R. Harvey, *J. Cell. Biol.*, **31**, 107 (1966).
15) Y. Inamori, S. Sunagawa, M. Tsuruga, Y. Sawada, H. Taniyama, G. Saito and K. Daigo, *Chem. Pharm. Bull.*, **26**, 1147 (1978).