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The Mechanisms of Delayed Insecticidal Action of Streptothricin Antibiotics. I.[1] Toxic Symptoms and Distribution of Racemomycin-D into the Tissues of the 5th Instar Larvae of Silkworm, *Bombyx mori* LINNE

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Racemomycin-D showed strong delayed insecticidal action against the 5th instar larvae of silkworm, *Bombyx mori*. The pattern of toxic action was similar to that seen in mammals. In order to clarify the mechanism of insecticidal action of racemomycin-D, the authors investigated the distribution of racemomycin-D into the tissues of the larvae. In groups of larvae given various doses of racemomycin-D (50—200 μg/g), the concentration of racemomycin-D in the blood decreased gradually in all cases, but the decrease rate was lower than that found in mammals. Racemomycin-D was not excreted in the feces. Racemomycin-D was accumulated in larger quantities for a longer time in the Malpighian tubules than in any other tissue. Change of color of the Malpighian tubules was observed visually. These phenomena resemble the findings in mouse and rat described in a previous report, namely, in the mammals, racemomycin-D was accumulated in large quantities for a long time in the kidney, and the organ turned white.

Keywords——racemomycin-D; silkworm 5th instar larvae, *Bombyx mori*; insecticidal delayed action; Malpighian tubules; toxic symptoms; racemomycin-D level in blood; racemomycin-D distribution into tissues

Racemomycin-D is one of the streptothricin antibiotics produced by *Streptomyces lavendulae* OP-2.[2] Previously we reported that racemomycin-D had a broad-spectrum insecticidal activity.[3] We further reported[4] recently that other racemomycin compounds and citromycin, one of the streptothricin-like antibiotics, also showed insecticidal effects. These streptothricin antibiotics showed delayed insecticidal action against the adults of *Blattella germanica*. The time of onset of delayed toxicity was similar to that in mammals.[2] However, the mechanisms of insecticidal action of streptothricin antibiotics have not been clarified yet.

In this report, the toxic symptoms and distribution of racemomycin-D into the tissues of the 5th instar larvae of silkworm, *Bombyx mori*, were investigated from a comparative biological standpoint in order to obtain basic information for further studies on the mechanism of insecticidal action.

Materials and Methods

Antibiotic——The experiments were carried out using racemomycin-D[2] produced by *Streptomyces lavendulae* OP-2[5] then extracted and purified by the method described previously.[2]

Insect——The 5th instar larvae of silkworm, *B. mori* L. (body weight, 2.5—3.1 g) were used. Each dosage group consisted of 15 larvae.

Dose——The antibiotic was dissolved in 0.85% NaCl and this solution was injected into the larvae. For the control group, 0.85% NaCl solution was injected. In the toxicity experiment, doses were 200, 150, 100 and 50 μg/g. For the investigation of the distribution of racemomycin-D into tissues, 150 μg/g was injected; this dosage was about equal to the median lethal dose at 72 h after injection (Table I).

Treatment of the Larvae——Two μl of 0.85% NaCl containing racemomycin-D was injected into the larvae intraperitoneally through the 8th body segment using a microsyringe. A mixture of paraffin and vaseline (1:1) was applied to the injected part to prevent leakage of the blood immediately after removal of the

needle. The treated silkworms were kept in Petri dishes (9 cm diameter), and supplied with mulberry leaves.

Examinaion of Feces———Feces in the Petri dish in which the treated larvae had been kept were collected every 24 h and their weight was measured. Then, 2 g of the feces was homogenized in 20 ml of distilled water. The homogenate was centrifuged, and the supernatant was assayed microbially to determine the amount of racemomycin-D contained in the feces.

Concentration of Racemomycin-D in the Blood———Abdominal appendages of the treated larvae were removed and the blood was collected at the prescribed time after the injection. The concentration of racemomycin-D in the blood was determined by means of antimicrobial assay.

Organ Sample———The treated larvae were anesthetized with CO_2, and their ventriculus, small intestine, colon, rectum, fat body and the Malpighian tubules were excised. These tissues were washed with 0.85% NaCl solution, and the weight of each tissue was measured. The homogenate of each tissue was bioassayed to determine racemomycin-D.

Antimicrobial Assay———Antimicrobial activity was determined by means of the paper disk method employing an agar plate seeded with *Bacillus subtilis* PCI-219 (10^6 cell/ml). The plate was stored at 5°C for 2 h and then incubated at 37°C for 18 h. Amount of racemomycin-D was calculated from a calibration curve.

Temperature———Every experiment was carried out at 23.5±1.5°C.

Results

I. Toxicity and Symptoms

Mortality and General Findings———After administration of racemomycin-D (50—200 μg/g), symptoms and mortality in the silkworm were observed.

Spontaneous movement was little different from that of the control group until 24 h after drug administration. After 48 h some of the larvae had died, and survivors in every group showed reduced movement and decreased ingestion of mulberry leaves. The skin color changed from white to yellow-brown and the skin became less lustrous. At 72 h the intersegmental membrane of the skin was black, and the groups given 100, 150 and 200 μg/g ingested little. The larvae were immobilized and vomited yellow fluid.

The mortality count is shown in Table I. Deaths occurred in the group given 100 μg/g after 48 h and in the group given 50 μg/g after 72 h.

Body Weight Change———The results are shown in Fig. 1. Up to 24 h, the body weight in the dosed groups increased, though the rate was less than that of the control group. However, after 24 h the weights of the larvae given 150 and 200 μg/g decreased remarkably and the weights in the groups given 50 and 100 μg/g also decreased.

Amount of Feces———After administration of racemomycin-D, the amount and the appearance of feces were examined. The result is shown in Fig. 2. The amount of feces decreased with time in each of the administered groups. The rate of decrease was especially large after 72 h. The feces of the administered groups became softer from 72 h after injection.

II. Distribution of Racemomycin-D

In Blood and Feces———The concentration of racemomycin-D in the blood was examined. The results are shown in Fig. 3. The amount of racemomycin-D in the blood

TABLE I. Time Course of Mortality of Silkworm, *Bombyx mori*, injected with Racemomycin-D

Dose (μg/g)	% mortality				
	24 h	48 h	72 h	96 h	120 h
200	0	20.0	80.0	100.0	100.0
150	0	6.7	46.7	100.0	100.0
100	0	6.7	33.3	93.3	100.0
50	0	0	13.3	73.3	86.7
Control	0	0	0	0	0

Temperature: 23.5±1.5℃.
Experimental size: 15 insects/group (2 groups).

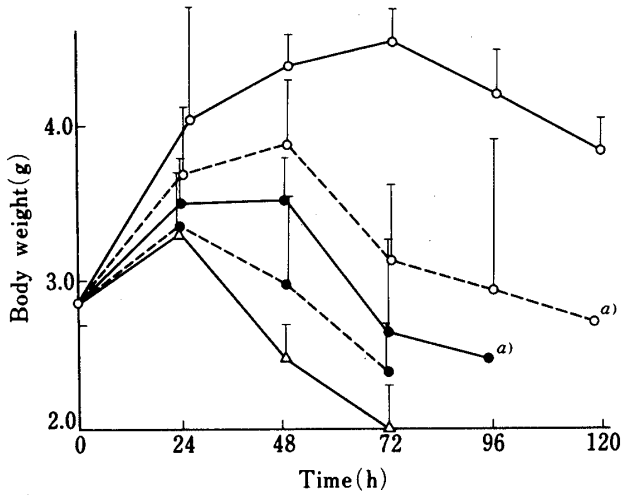

Fig. 1. Body Weight Change in the 5th Instar Larvaeof Silkworm, *Bombyx mori*, administred Racemomycin-D

a) 1 silkworm had died due to strong delayed toxicity. —○—, control; dose --○--, 50 μg/g; —●—, 100 μg/g; --●--, 150 μg/g; —△—, 200 μg/g. Body weight of silkworms used in the experiments was 2.839±0.147 g.

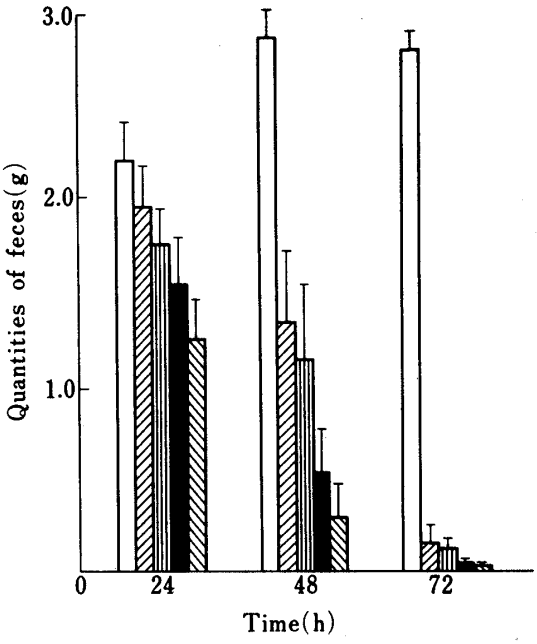

Fig. 2. The Quantity of Feces of the 5th Instar Larvae of Silkworm, *Bombyx mori* after Administration of Racemomycin-D

Results are mean values (± SD, 3—15 silkworms). □, control; dose ▨, 50 μg/g; ▥, 100 μg/g; ■, 150 μg/g; ▧, 200 μg/g.

decreased gradually after the treatment. However, the compound was still present, even in the larvae treated with 50 μg/g, at 96 h after injection.

Secondly, the concentration of racemomycin-D in feces was examined. However, racemomycin-D was not detected even at 24 h after injection.

In Tissues——The distribution of racemomycin-D into the tissues was investigated. The results are shown in Table II. Larger quantities of racemomycin-D were distributed for a longer time in the Malpighian tubules than in any other tissue. At 72 h after administration, the color was black (visual observation).

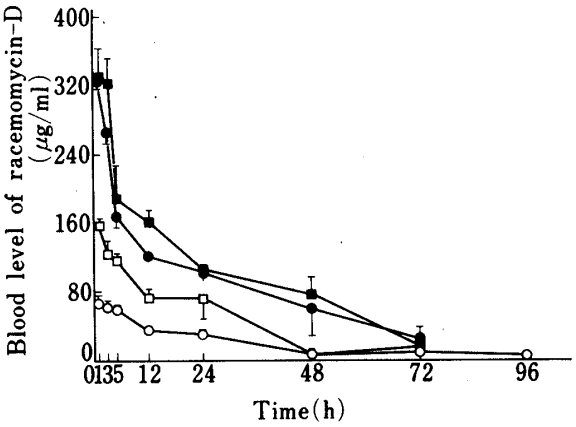

Fig. 3. Apparent Blood Level of Racemomycin-D based on Antimicrobial Activity Assay

Results are mean values (± SD) for groups of 3 silkworms.
—■—, 200 μg/g; —●—, 150 μg/g; —□—, 100 μg/g; —○—, 50 μg/g.

Discussion

Racemomycin-D showed strong delayed toxicity against silkworm larvae (Table I). The pattern of the toxic action was very similar to that observed in the adults of *Blattella germanica*[3] and in mice.[2] The changes of the body weight were similar in principle to those in mice[6] (Fig. 1).

As a preliminary step to investigate the mechanisms of insecticidal action of the antibiotic, its distribution into the tissues of insects was examined using the antimicrobial assay method.

In every group given racemomycin-D, the blood level decreased with time (Fig. 3). No blood sample from the 100 μg/g group was available at 96 h after injection because the mortality was 100% or close to this value. However, in the group given 50 μg/g racemomycin-D, the drug was still detectable in the blood even at 96 h after administration. In contrast, in mice and rats, the antibiotic had disappeared by 2 h after injection. This difference of blood level is presumably due to the different metabolic activities of insects and mammals.

Next, the excretion of racemomycin-D should be considered. The amount of feces excreted by larvae after drug administration decreased with time relative to that of the control

TABLE II. Distribution of Racemomycin-D in *Bombyx mori* Tissues after Administration

Tissues	Residual antimicrobial activity (μg/g) Time after administration (h)						
	1	3	5	12	24	48	72
Malpighian tubule	33.49	37.89	41.14	88.12	88.12	64.44	113.45
Ventriculus	15.42	13.63	11.33	11.88	16.75	20.46	16.01
Small intestine	11.80	16.98	10.72	11.09	12.05	10.63	10.60
Colon	9.81	11.72	14.96	12.05	10.01	6.55	10.20
Rectum	19.88	27.07	25.51	18.56	21.00	10.66	23.20
Fat body	18.56	33.17	27.26	27.82	40.58	39.48	52.67

Bioassay: paper disk method using *B. subtilis* PCI-219.
Values are means of 30 silkworms.
Dose: 150 μg/g.
Route: injection.

group (Fig. 2). The decrease of feces suggests a toxic effect of racemomycin-D on the digestive system.

The authors have reported that after administration of racemomycin-D the amount of urine of rats[7] decreased remarkably. However, in the case of the silkworm it is not clear whether racemomycin-D is excreted in the feces, or, if it is excreted, whether it is in an inactivated form. To clarify this the labelled compound should be used.

As for the distribution of racemomycin-D into the tissues after administration, larger quantities of racemomycin-D were distributed in the Malpighian tubules for a longer time than in any other tissue (Table II). Further, the Malpighian tubules changed color. The tubules are the excretory organ of the insects and their function could be equivalent to that of the mammalian kidneys. In mammals, streptothricin antibiotics show strong nephrotoxicity,[8] and we recently showed that: 1) a large quantity of racemomycin-D is accumulated in the kidneys of mice[6] and rats,[9] 2) the kidney turns white, 3) strong nephrotoxicity[7,9] is found. It is interesting that although differences exist between insects and mammals biochemically, physiologically, and morphologically, racemomycin-D is accumulated in the excretory organs in both animals.

Since a large quantity of racemomycin-D was accumulated in the Malpighian tubules and an abnormal change of the color was observed, histopathologic examination of the Malpighian tubules is required. For the following reasons, we consider that the mechanism of insecticidal action of streptothricin antibiotics involves its deteriorating action on the excretory organs.

1) Its action is not acute but delayed.

2) The excretion decreases with time and the feces become soft (this phenomenon has also been observed in the adults of *Blattella germanica*[10]).

3) Racemomycin-D does not inhibit the nervous system or the energy metabolism system.[11]

4) Large quantities of racemomycin-D accumulated in the Malpighian tubules for a long time, and the tubules changed color. On the other hand, in the case of mammals it is distributed into the kidney in a large quantity for a long period, and shows strong nephrotoxicity.

Further histopathologic and biochemical investigations are in progress.

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11) Research on the effect of streptothricin antibiotics on the nervous system and energy metabolism system will be reported in the next paper.