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Changes of Tissue Histopathology and Uric Acid Excretion in Silkworm Larvae Intoxicated with Deoxypodophyllotoxin and Racemomycin-D, and during Starvation

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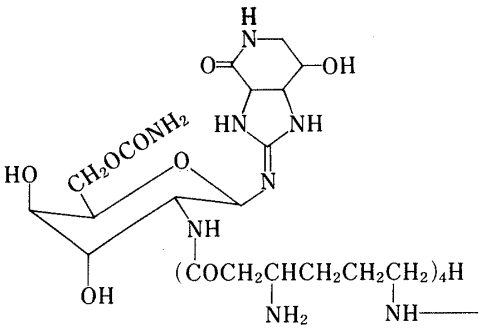
Histopathological examination of tissues and analysis of uric acid in hemolymph and feces of the 5th instar larvae of the silkworm, *Bombyx mori* LINNE, either treated with racemomycin-D or deoxypodophyllotoxin both of which showed delayed insecticidal activities, or being starved, gave the following results. 1) In the larvae treated with racemomycin-D the epidermal cells and fat body cells were seriously damaged along with the Malpighian tubules, 2) in the larvae treated with deoxypodophyllotoxin, the fat body cells as well as the epidermal cells were seriously damaged, but the Malpighian tubules remained intact, 3) on starvation, only the fat body cells were damaged. 4) Both in the starved and the deoxypodophyllotoxin-treated larvae, a considerable increase of uric acid was noted in hemolymph and feces.

It was concluded that the increase of uric acid in hemolymph and feces was caused by the histolysis of fat body cells due to inadequate feed intake, resulting either directly through starvation or indirectly through the effects of the chemicals.

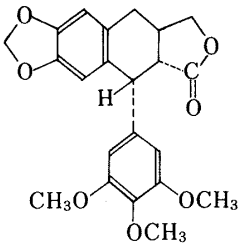
Keywords——deoxypodophyllotoxin; racemomycin-D; epidermal cell; fat body; Malpighian tubule; silkworm; *Bombyx mori*; uric acid; starvation

Uric acid is the main end-product of nitrogen catabolism in many insects. Its relation to intoxication in insects has not been studied as extensively as in animals, and only a few reports have appeared. In the larvae of the silkworm, *Bombyx mori*, fed with mulberry leaves of inferior quality, uric acid excretion increased.[1] Further, the Malpighian tubules were seriously damaged and uric acid increased in both hemolymph and feces on treatment of the larvae with racemomycin-D (RM-D, Fig. 1),[2] a streptothricin antibiotic with insecticidal activity. It was assumed by these authors that the results corresponded to those in rats treated with RM-D, whose blood level of urea increased as a result of the strong nephrotoxicity.[3] However, there are many differences between insects and mammals, and it is difficult to draw conclusion simply on the basis of an apparent similarity of reactions. It has also been reported that deoxypodophyllotoxin (DPT, Fig. 1),[4] a lignan isolated from the root of *Anthriscus sylvestris* HOFFM, showed strong insecticidal activity accompanied with damage to the epidermal cells of silkworm larvae.[5-7] No detailed work, however, has been done on the relationship between the tissue damage and the increase of uric acid in hemolymph and feces of silkworm larvae on treatment with DPT.

Therefore, in order to clarify the relation of uric acid with intoxication in the 5th instar larvae of *B. mori*, we carried out histopathologic examinations of tissues other than the Malpighian tubules and analyzed uric acid in hemolymph and feces after treatment with RM-D and DPT, and compared the changes with those during starvation.



racemomycin-D (RM-D)



deoxypodophyllotoxin (DPT)

Fig. 1. Chemical Structures of Racemomycin-D and Deoxypodophyllotoxin

Materials and Methods

Chemicals——RM-D and DPT were used. RM-D was isolated from the culture broth of *Streptomyces lavendulae* OP-2[8] according to the method of Inamori *et al.*[9] DPT was isolated from the root of *Anthriscus sylvestris* HOFFM by the authors.[4]

Insects——The 5th instar larvae of silkworm, *B. mori* (body weight, 2.5—3.1 g) reared on fresh mulberry leaves were used. The temperature was kept at 23.5±1.5 °C throughout the experiments.

Treatment of Larvae——RM-D was dissolved in 0.85% NaCl aqueous solution at a dose of 150 μg/g and 2 μl of the solution was injected into the hemocoele of each larva intraperitoneally through the 8th abdominal segment using a microsyringe, as in the previous papers.[2,5] A mixture of liquid paraffin and vaseline (1 : 1) was applied to the injected part to prevent leakage of hemolymph immediately after removal of the needle. The larvae were kept in Petri dishes (9 cm diameter), and fed on fresh mulberry leaves. As the control, 2 μl of 0.85% NaCl solution was injected. DPT was dissolved in acetone at a concentration of 1000 ppm. The mulberry leaves were dipped in the solution, dried at room temperature, and given to the larvae in Petri dishes as in the previous paper.[6] The control animals were fed on leaves treated only with acetone. For the starvation experiment, the larvae were kept in Petri dishes without leaves.

Histopathological Examination——At various intervals after chemical treatment or starvation, the larvae were anesthetized with CO_2 and the integument, fat body, Malpighian tubules and ventriculus were dissected out. These tissues were washed with 0.85% NaCl solution, fixed in Bouin's solution (saturated picric acid 75 ml, formalin 25 ml and glacial acetic acid 5 ml) for 24 h, and sectioned at 5—6 μm thickness after being embedded in paraffin. They were stained with hematoxylin–eosin solution, and examined under an optical microscope at a magnification of 200.

Quantitative Analysis of Uric Acid in Hemolymph——The abdominal legs of DPT-treated or starved animals were cut off at various times and hemolymph was collected. The hemolymph was centrifuged at 10000 rpm for 3 min, and 50 μl of the serum was used for uric acid determination with a Determiner UA[10] (uricase–peroxidase method; Kyowa Hakko Co., Ltd.).

Quantitative Analysis of Uric Acid in Feces——Feces of the larvae were collected at the prescribed time after treatment and dried. Dried feces were weighed, reduced to powder, and extracted with water on a boiling water bath for 3 h. The juice was centrifuged at 10000 rpm for 3 min and 50 μl of the supernatant was assayed for uric acid as mentioned above.

Results

Histopathological Changes of the Fat Body and Epidermal Cells in Larvae Treated with Racemomycin-D

Along with the Malpighian tubules, which have been shown to be seriously damaged by RM-D treatment,[2] the fat body and epidermal cells were histopathologically examined at various times after injection of RM-D. As shown in Fig. 2a, although the amount of oil droplets in the fat body cells of the treated group was almost equal to that of the control group at 12 h, large vacuoles were seen prominently in former group, possibly caused by fusion of the oil droplets. The fine structure of the tissues at 24 h seemed to be almost the same as that at 12 h (Fig. 2b). However, a further 24 h later, considerable coagulation of chromatin in the nuclei of the treated animals, which might be a symptom of pyknosis, was apparent. The number of vacuoles in the treated group became fewer, and all of the cells began to

atrophy (Fig. 2c). At 72 h the chromatin showed marked coagulation and the basement membrane became thinner (Fig. 2d). Oil droplets were hardly found, most of the vacuoles had disappeared, and atrophy of the cells was apparent.

The epidermal cells did not differ in structure from those of the control group at 24 h (Fig. 3a). However, after 48 h the cuticles were considerably swollen and a large number of granules appeared on the side of the body cavity. The epidermal cells began to collapse and the granules, stained with hematoxylin and eosin, showed coagulation (Fig. 3b). At 72 h, the cells of the treated animals were almost necrotized (Fig. 3c). RM-D treatment caused no damage to the ventriculus, as has already been shown by us.[2)]

Histopathological Changes of the Fat Body Cells in Larvae Treated with Deoxypodophyllotoxin

Epidermal cells were severely damaged by DPT treatment, as described in previous report.[7)] Here, we further histopathologically checked the changes of the fat body cells after treatment of DPT. As shown in Fig. 4a, up to 24 h, the fat body cells looked normal. However, after 48 h, these cells were considerably atrophied and there were fewer oil droplets in the cytoplasm (Fig. 4b). At 72 h, atrophy was clearer and oil droplets were hardly found (Fig. 4c). DPT caused no damage to the Malpighian tubules or ventriculus (data not shown).

Histopathological Changes of the Fat Body Cells in Larvae during Starvation

The fat body cells were examined histopathologically at various times during starvation. As shown in Fig. 5a, the oil droplets in the fat body cells were decreased in number at 12 h and atrophy could be recognized in all of the cells. At 24 h the oil droplets were further decreased and in the cytoplasm only proteinaceous structure could be recognized (Fig. 5b). At 48 h the oil droplets were still further decreased and the nuclei were severely atrophied (Fig. 5c). At 72 h the oil droplets were almost absent and the nuclei were further shrunken, although the nuclear membranes seemed to be intact (Fig. 5d). These observations demonstrated that starvation caused serious damage to the fat body cells. The epidermal cells, the Malpighian tubules and ventriculus were not damaged by starvation, and looked normal, as in the fed control (data not shown).

Changes of Uric Acid Levels in Hemolymph and Feces in Larvae Treated with Deoxypodophyllotoxin or Starved

The effects of DPT and starvation on the levels of uric acid in hemolymph and feces were investigated. As shown in Table I, the concentration of uric acid in hemolymph was higher than in the fed control at 12 h after the start of starvation. At 72 h, it became six times higher than the control. In the larvae treated with DPT, the level of uric acid was higher than that of the fed control at 12 h, and at 72 h attained a level twenty-five times higher than the control.

Further, the excretion of uric acid was compared as shown in Table II. The concentration of uric acid in feces also increased steeply at 12 h after the start of starvation, and at 48 h attained a level eighty-five times higher than the control. In the animals treated with DPT, the level of uric acid increased within 12 h. At 24 h, it was eight times higher than the control. However, feces were not excreted because of the strong delayed toxicity of DPT at 48 h.

Discussion

The authors[2)] previously reported that there was a relation between the damage to the Malpighian tubules and the increase of uric acid in hemolymph and feces of silkworm larvae treated with RM-D. However, this conclusion is not supported by the following three results in the present paper. 1) In DPT-treated larvae, the Malpighian tubules were not destroyed, while the epidermal cells and fat body cells were certainly damaged, and uric acid was increased in both hemolymph and feces. 2) RM-D also caused severe damage in both the

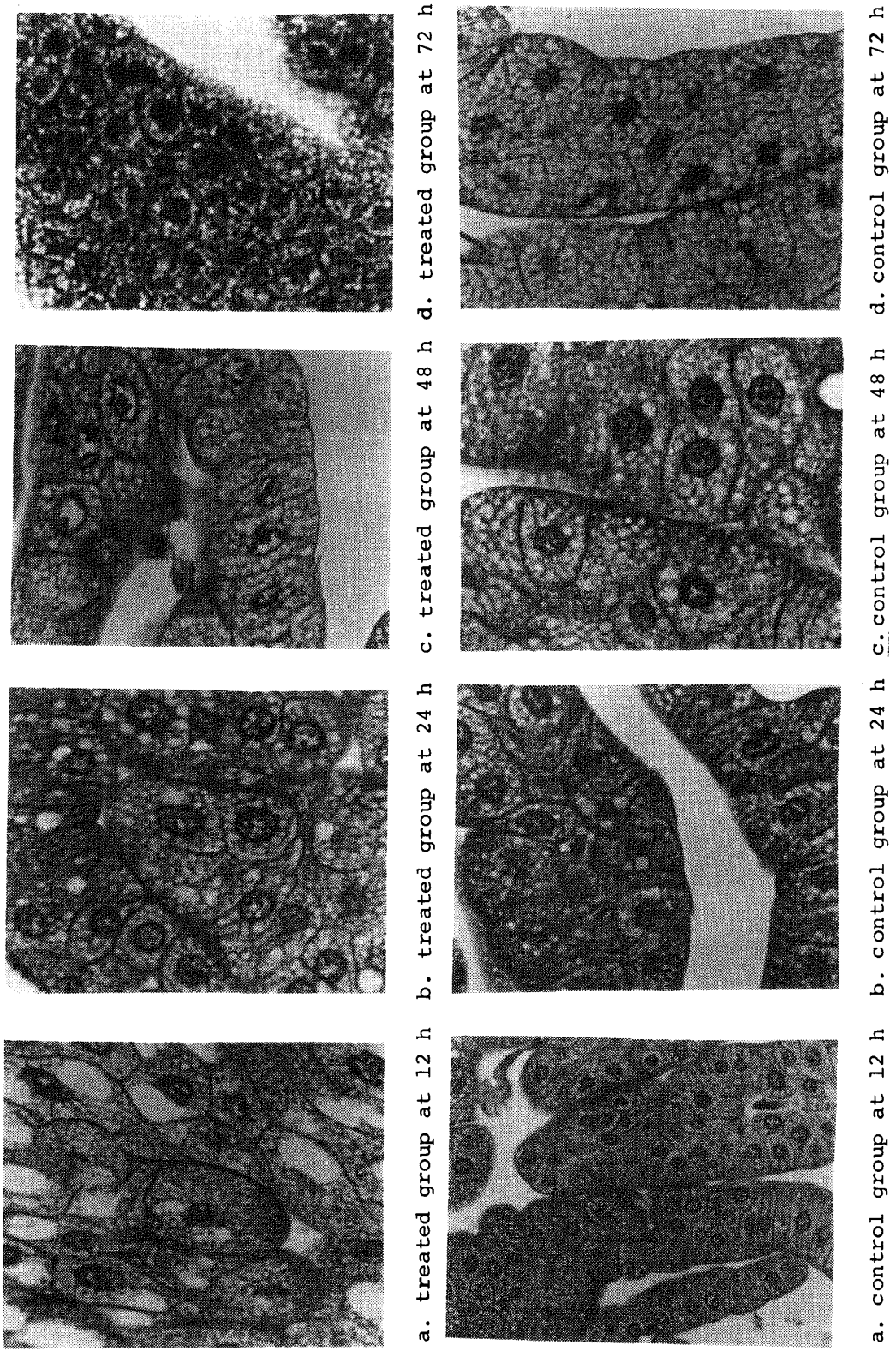


Fig. 2. Photographs of the Fat Body Cells of the 5th Instar Larvae of Silkworm, *B. mori*, at Various Times after Treatment with Racemomycin-D (Hematoxylin–Eosin Staining × 200)

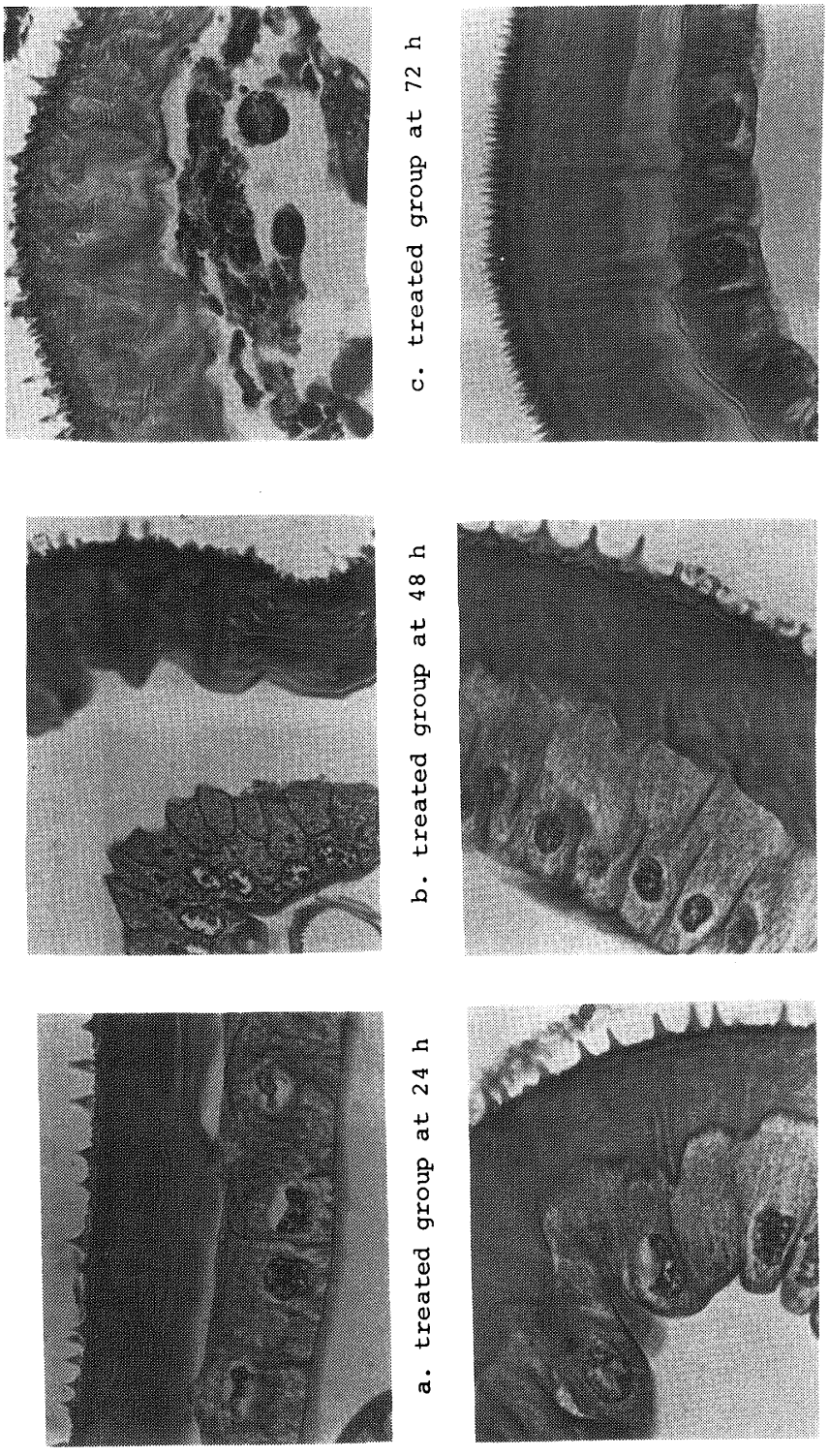


Fig. 3. Photographs of the Epidermal Cells of the 5th Instar Larvae of Silkworm, *B. mori*, at Various Times after Treatment with Racemomycin-D (Hematoxylin–Eosin Staining × 200)

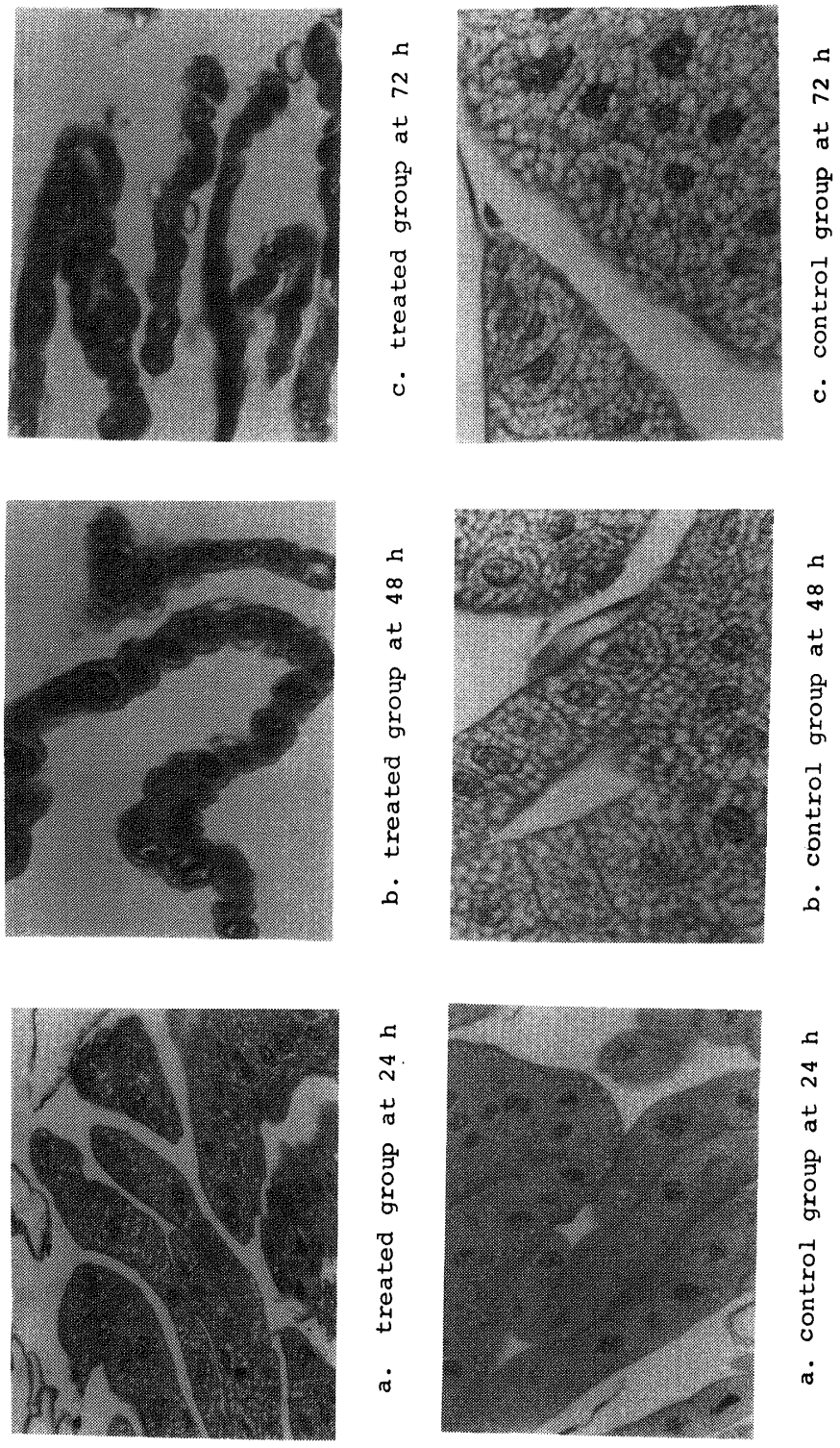


Fig. 4. Photographs of the Fat Body Cells of the 5th Instar Larvae of Silkworm, *B. mori*, at Various Times after Treatment with Deoxypodophyllotoxin (Hematoxylin–Eosin Staining ×200)

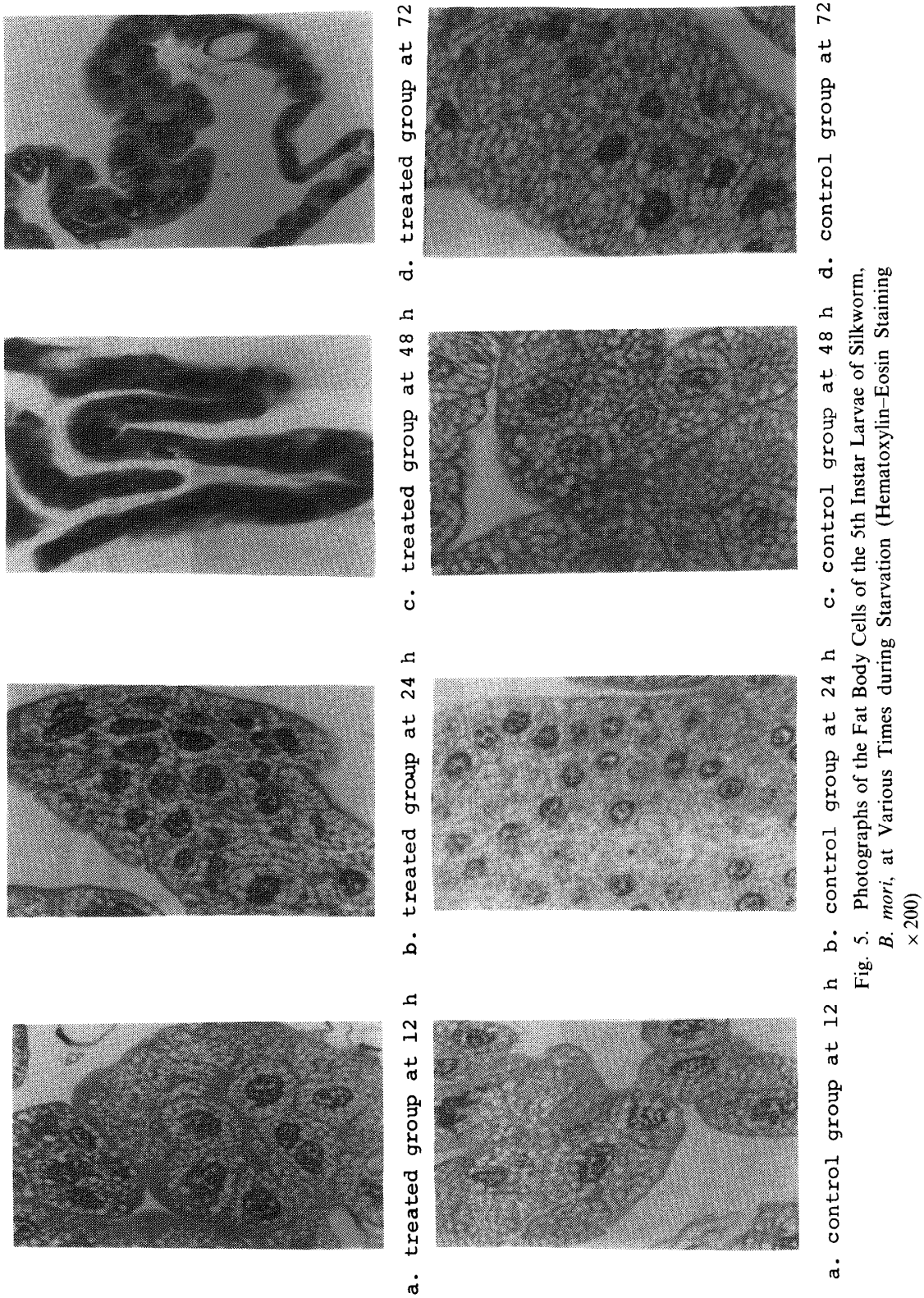


Fig. 5. Photographs of the Fat Body Cells of the 5th Instar Larvae of Silkworm, *B. mori*, at Various Times during Starvation (Hematoxylin–Eosin Staining ×200)

TABLE I. Changes of Uric Acid Concentration in Hemolymph of the 5th Instar Larvae of *B. mori* Treated with Deoxypodophyllotoxin,[a] or Starved

Time (h)	DPT-treated	Starved	Control
12	18.87±2.42	6.13±0.22	1.80±0.35
24	7.32±1.11	6.18±0.49	2.64±0.61
48	22.29±2.52	7.45±0.34	1.68±0.17
72	43.86±5.46	11.02±0.39	1.78±0.10

Each value represents the mean±S.D. ($n=5$). Assay method: Uricase–peroxidase method. Unit: mg/dl. *a*) The larvae were fed on mulberry leaves dipped in DPT solution (1000 ppm).

TABLE II. Changes of Uric Acid Concentration in Feces of the 5th Instar Larvae of *B. mori* Treated with Deoxypodophyllotoxin,[a] or Starved

Time (h)	Treated group	Starved	Control
12	3.28±0.43	4.10±0.69	1.03±0.12
24	18.43±5.21	43.15±2.24	2.32±0.57
48		68.92±1.34	0.81±0.28
72		66.74±5.03	0.36±0.18

Each value represents the mean±S.D. ($n=5$). Assay method: Uricase–peroxidase method. Unit: mg/dl. *a*) The larvae were fed on mulberry leaves dipped in DPT solution (1000 ppm). Larvae treated with DPT did not excrete feces because of strong delayed toxicity at 48 h.

epidermal and fat body cells. 3) Starvation caused severe damage only in the fat body cells, and also induced an increase of uric acid in hemolymph and feces. Although in a previous paper[2] the increase of uric acid then assumed to be related to damage to the Malpighian tubules was suggested to be analogous to the increase of urea in the blood in mammals, both being caused by the nephrotoxicity, this has now been shown to be incorrect.

The fact that damage to the fat body and increase of uric acid in hemolymph and feces occur in essentially similar ways during both starvation and intoxication supports the idea that the test chemicals inhibit feeding, which might induce damage to the fat body, indireclty stimulating uric acid excretion. Biochemical studies on the relationship between the increase of uric acid in hemolymph and feces and the damage to the fat body cells are in progress.

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