

Effects of Two Chemosterilants, Metepa and Hempa, on the Hemolymph Proteins in the Last Instar Larvae and Pupae of the Smaller Citrus Dog, *Papilio xuthus* LINNE.¹ Isamu NAKAYAMA*, Tadaharu KITAGAKI* and Ken'ichi KOJIMA** (Biological Research Laboratory, Kumiai Chem. Ind. Co., Ltd., Toxicological and Animal Health Laboratory, Kumiai Chem. Ind. Co., Ltd., ** Kikugawa, Shizuoka-Pref.) Received April 19, 1971. *Botyu-Kagaku* 36, 105, (1971)

15. アゲハ終令幼虫体液蛋白におよぼす metepa および hempa の影響 中山 勇*, 北垣忠温*, 小島建一** (クミアイ化学工業株式会社, 生物研究所*, クミアイ化学工業株式会社, 動物研究所**) 46. 4. 19. 受理

アゲハ終令幼虫および蛹の体液蛋白におよぼす metepa および hempa の影響を、寒天ゲル電気泳動法をもちいて検討した。

幼虫体液蛋白について、無処理の終令4日目の幼虫では、A₁, A₂, G₁ および G₂ の4分画が見出されたが、metepa および hempa の 4,000 μ g/幼虫投与群では G₁ 分画の消失および G₂ 分画の減少が認められた。また metepa の 1,000 μ g/幼虫投与群において G₁ 分画の減少がみられたが、同葉量投与群の hempa のそれは無処理と大差なかった。G₂ 分画の減少は hempa でより著しかった。蛹化1日後の体液蛋白の G₂ 分画は metepa より hempa 処理において顕著な減少を示した。

幼虫および蛹期の精巢ホモジネート上清には 1 蛋白分画のみ見い出された。本分画におよぼす不妊剤の影響は必ずしも明瞭でなかった。

無処理終令4日後の幼虫および蛹の A/G 比は、それぞれ 1.54 11.50~11.82 であった。A/G 比は処理区で変動を示したが、それは G₁ および G₂ 分画の減少に起因するものと考えられる。

Introduction

In recent years, the effect of chemosterilants on the external form of pupae in Lepidoptera has been mainly investigated on the silkworm, *Bombyx mori* L. (Hirano 1965,¹⁾ Sugai and Hirano, 1967²⁾. Hirano (1965)¹⁾ reported that degeneration of antennae, legs and wings in deformed pupae, were induced by apholate administered orally to larval stages of the silkworm.

Similar results were also obtained in this laboratory using pupae of the smaller citrus dog, *Papilio xuthus* L., and the gypsy moth, *Porthetria (Lymantria) dispar* L., in which metepa was administered orally to insects in the last larval instar. However, no deformed pupae were observed when hempa was administered orally to the smaller citrus dog (Unpublished data). The results of this work suggest that there may be differences in the action of these two chemosterilants to some insects.

The present investigation was undertaken to

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investigate whether the two chemosterilants have a similar effect on the hemolymph proteins of the last instar larvae and pupae of the smaller citrus dog.

Materials and Methods

Chemicals and Applications; Metepa (Tris (2-methyl-1-aziridinyl)-phosphine oxide) and hempa (Hexamethylphosphoric triamide) were dissolved in distilled water at concentrations of 1,000 μ g, 4,000 μ g and 8,000 μ g per 0.1 ml immediately prior to treatment.

Insect; Female adults of the smaller citrus dog were collected from the field in Kikugawa and reared in the laboratory at a temperature of 25°C, relative humidity of 60% and all day illumination (24 hour light). Eggs of these adults were reared on leaves of the mandarine orange, *Citrus unshiu* Marcos. Chemicals were administered orally to the larvae of fifth instar at several ages at a rate of 0.1 ml per larva by means of a microsyringe. Treated larvae were reared on the orange leaves until they were used for electrophoretic studies.

Electrophoresis; Hemolymph and testis were

collected from the male larva of 4 day-old, fifth instar larvae treated with chemicals. Hemolymph was obtained from 7th dorsal segment of the larvae (Kitagaki *et al.*, 1970²⁾). A pair of testes was removed as quickly as possible, was homogenized in 20 μ l of veronal buffer, pH 8.6 with a glass homogenizer, and the resulting homogenate was centrifuged at 8,000 rpm for 4 minutes. The supernatant was used for the electrophoretic investigations.

The electrophoresis was carried out as described by Kitagaki *et al.* (1970)²⁾, using the following buffer system and method.

Agar..... 400mg
 P. V. P (Polyvinylpyrrolidone, K-90).....200mg
 Veronal buffer, pH 8.6, $\mu=0.025$ 40ml
 Size of a glass plate..... 50 \times 200mm
 Thickness of gel layer..... 1mm
 Constant current..... 2.5mA/cm
 Time of electrophoresis..... 60min.
 Temperature of electrophoresis.....5°C

A sample of 2 μ l was applied to a short slit of 10mm width on the agar gel plate by means of a microsyringe and subjected to electrophoretic separation. Proteins were detected by Amido Black 10B staining under conditions recommended by Yushima and Kamano (1964)⁷⁾. Each fraction was determined by a densitometer, QUICK of Atago Optical Works Co., Ltd., using 650 μ and

a slit width of 8mm. Human serum (Moni-Trol 1, Dade Reagents Inc.) was used as a standard.

Results

As the first step, a relationship between the deformed pupae and concentrations of two chemosterilants was studied. When three different doses of metepa and hempa were orally administered to newly molted 5th instar larvae of the smaller citrus dog, abnormal pupae were classified into 8 ranks according to the degree of deformation of the pupa as shown in Table 1, that is 1, 2, ... 7 and normal pupa shown as 8.

As shown in Table 1, it was very clear that metepa treatment with doses higher than 4,000 μ g per larva greatly induced the formation of deformed pupa. Following treatment with 8,000 μ g of hempa the larvae did not grow and thereafter died. In order to elucidate the difference of action between metepa and hempa, as shown in Table 1, electrophoretic patterns of the proteins of hemolymph and testes from larvae and pupae orally treated with the two chemosterilants after the 4th moulting larva were investigated.

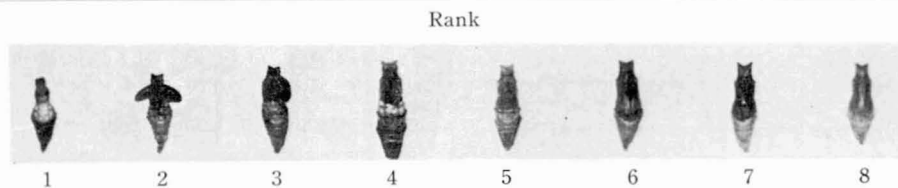
The effect on hemolymph proteins of larvae ;

In a previous paper, the authors (1970)²⁾ reported that main four protein fractions could be found in the hemolymph of untreated 4day-old,

Table 1. Numbers of deformed pupae of smaller citrus dogs.

Chemicals*	Dosage (μ g/larva)	Rank								No. of Larvae	No. of Kills
		1	2	3	4	5	6	7	8		
		Abnormal				Normal					
Hempa	8,000	0	0	0	0	0	0	0	0	10	10
	4,000	0	0	0	0	0	0	0	6	10	4
	1,000	0	0	0	0	0	0	0	7	10	3
Metepa	8,000	2	0	0	0	0	0	0	0	10	8
	4,000	1	3	2	1	0	0	0	0	10	3
	1,000	0	0	0	0	0	1	4	4	10	1

* Chemicals were orally administered at larvae.



fifth instar. These fractions were named A₁, A₂, G₁ and G₂, starting with the designation of A₁ for the most rapidly migrating component.

In this investigation, four main fractions were again detected in the hemolymph of untreated 4 day-old, fifth instar larvae. However, as shown in Figure 1, fraction G₁ decreased markedly and fraction G₂ decreased slightly, when 4,000 μg of hempa or metepa and 1,000 μg of metepa were orally administered to larvae immediately after the fourth moult. Following treatment with 1,000 μg of hempa the decrease of fraction G₁ was scarcely noticeable. Fraction G₂ in hempa decreased more than the metepa and untreated.

When 1 day-old, fifth instar larvae were treated with 4,000 μg or 1,000 μg of metepa per larva fractions G₁ and G₂ were more obvious than in the case of larvae treated immediately after the 4th moult. It is thought that these fractions might be the amount of protein which was accumulated in hemolymph before the treatment of metepa.

The effect on proteins in hemolymph of pupae ;

The electrophoretic patterns of proteins in the hemolymph of pupae following oral administration of 4,000 μg of metepa or hempa per larva immediately after the 4th larval moult are shown in Figure 2.

Fraction G₂ on the hemolymph of 1 day-old pupa decreased substantially were following hempa treatment than metepa treatment and was smaller than in the controls. Similar results were observed in 3 day-old pupa. In the pupal stage, only one fraction A was found. Fractions A₁ and A₂ which were detected in 4-day-old fifth instar larvae were not observed. There was no significant difference between the hempa and metepa treatments in the amounts of fraction A. However, this fraction decreased slightly compared to the untreated.

The effect on proteins in the soluble fraction of the testes ;

Electrophoretic patterns of proteins in soluble fraction of the testis obtained from 4 day-old

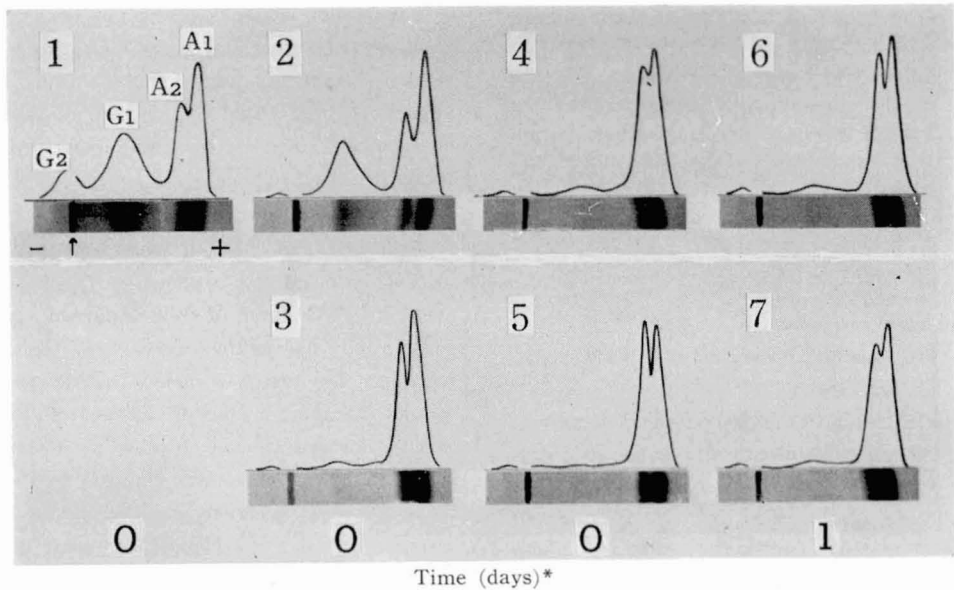


Fig. 1. Electrophoretic patterns of the hemolymph of larvae of smaller citrus dogs.

* Chemicals were orally administered to newly emerged or 1 day-old, fifth instar larvae.

The hemolymph was taken from 4 day-old fifth instar larvae.

- (1) Untreated
- (2) Hempa, 1,000 μg/larva
- (3) Hempa, 4,000 μg/larva
- (4 and 6) Metepa, 1,000 μg/larva
- (5 and 7) Metepa, 4,000 μg/larva

i insect chemosterilants.

Discussions

The above observations revealed that metepa and hempa treatment causes changes in the protein content and A/G ratios in the hemolymph of larvae and pupae of the smaller citrus dog. Palmquist and Lachance (1966)⁴⁾ demonstrated a sterilization effect in the parasitic wasp, *Bracon hebetor* Say (Habrobracon), treated with the alkylating agent, tepa and its nonalkylating analog, hempa. These compounds induced a high frequency of recessive lethal mutations in the sperm of this insect, although tepa was the more efficient mutagen. Sugai and Hirano (1967)⁶⁾ reported that apholate inhibited markedly the growth of the wings in pupae of the silkworm, *Bombyx mori* Linnae. Sharma and Rai (1969)⁵⁾ also suggested that there are similarities between the action of apholate and the synthetic juvenile hormone.

On the other hand, Kitagaki *et al.* (1970)²⁾ reported that G₁ and G₂ fractions in hemolymph of the smaller citrus dog were corresponded to globulins in the rice stem borer, *Chilo suppressalis* Walker, hemolymph and human serum. Koike (1962)³⁾ suggested that G₁ fraction of globulins in the rice stem borer have an important physiological function.

The A/G ratios of untreated 4 day-old, fifth instar larvae and of pupae were 1.54 and 11.50 to 11.82, respectively, as shown in Table 2. However, the A/G ratio of treated larvae changed substantially due to the decrease of G₁ and G₂ fractions. The effect of metepa on the globulin content was more marked than that from hempa, at high dosages. The A/G ratio in hemolymph of hempa-treated pupae was changed by the decrease of the G₁ fraction and the increase of A fraction, although the hempa treatment did not induce any deformed pupae.

From these results, it is suggested that metepa and hempa may block the synthesis of the G₁ and G₂ protein fractions in this insect. Metepa was more effective than hempa in decreasing the G₁ fraction but hempa was more effective than metepa with respect to decreasing the G₂

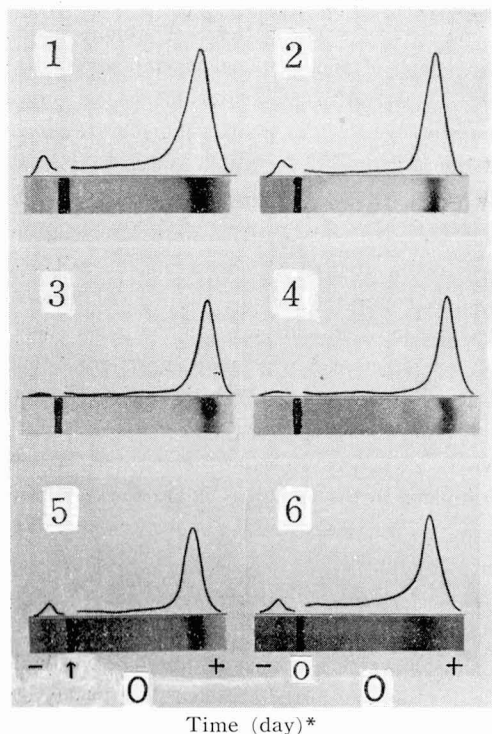


Fig. 2. Electrophoretic patterns of the protein in the hemolymph of the pupae of smaller citrus dogs.

* Chemicals were orally administered to newly moulted fifth instar larvae, at a rate of 4,000 μ g/larva. The hemolymph was taken from 1 day-old (left) and 3 day-old (right) pupae.

- (1 and 2) Untreated
- (3 and 4) Hempa
- (5 and 6) Metepa

larvae fifth instar larvae and 3 day-old pupae are shown in Figures 3 and 4.

In this investigation, 4,000 μ g of metepa and hempa were administered orally to larvae immediately after the 4th moult. One day-old fifth instar larvae were also treated with 4,000 μ g and 1,000 μ g of metepa. Only one fraction was found in the soluble fraction of the testis in both larval and pupal stages. This fraction did not differ in treated and untreated larvae, and on the nature and properties of this protein are unknown. Consequently it is necessary to carry out a more detailed study of the proteins of the testes in insects, with reference to the mode of action of

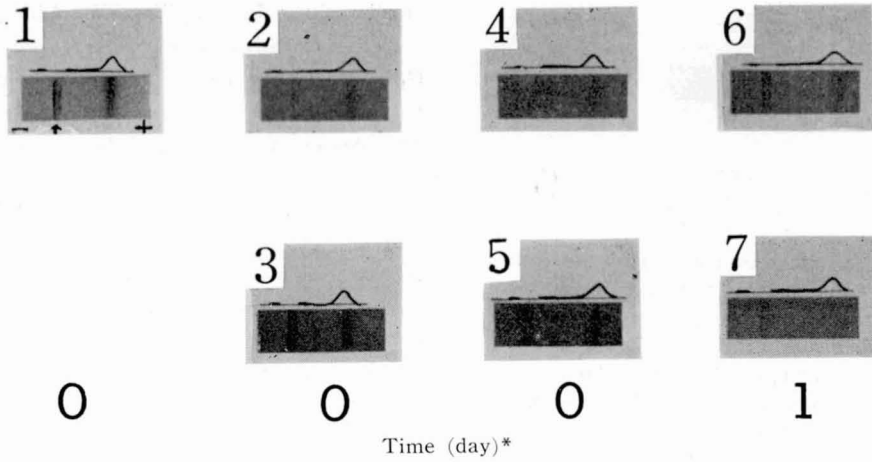


Fig. 3. Electrophoretic patterns of the protein from the soluble fraction of larval testes of smaller citrus dogs.

* Chemicals were orally administered to newly emerged or 1 day-old, fifth instar larvae.

The testis was taken from 4 day-old fifth instar larvae.

- (1) Untreated
- (2) Hempa, 1,000 μ g/larva
- (3) Hempa, 4,000 μ g/larva
- (4 and 6) Metepa, 1,000 μ g/larva
- (5 and 7) Metepa, 4,000 μ g/larva

Table 2. Protein electrophoresis of larval and pupal hemolymph of smaller citrus dogs.

Chemicals*	Dosage (μ g/larva)	Time of Treatment	Time Collected Hemolymph	Days from Treatment of Collected Hemolymph	Percent of Total Protein				A/G Ratio
					A ₁	A ₂	G ₁	G ₂	
Untreatment	0	0**	4***	4	22.6	38.0	34.4	5.0	1.54
Hempa	4,000	0	4	4	35.6	54.3	9.7	0.4	8.90
	1,000	0	4	4	22.6	41.7	31.4	0.3	2.15
Metepa	4,000	0	4	4	50.2	48.2	0.8	0.8	61.50
	1,000	0	4	4	42.1	46.8	9.7	1.4	8.01
	4,000	1	4	3	44.6	47.2	6.8	1.4	11.20
	1,000	1	4	3	38.4	47.5	12.8	1.3	6.09
	4,000	3	4	1	29.8	51.4	13.6	5.2	4.32
	1,000	3	4	1	30.7	49.0	14.2	6.1	3.93
Untreatment	0	0	1****	6	92.2		0.0	7.8	11.82
Hempa	4,000	0	1	6	100.0		0.0	0.0	—
Metepa	4,000	0	1	6	85.7		0.0	14.3	5.99
Untreatment	0	0	3	9	92.0		0.0	8.0	11.50
Hempa	4,000	0	3	9	99.0		0.0	1.0	99.00
Metepa	4,000	0	3	9	89.2		0.0	10.8	8.26

* Chemicals were orally administered at larval stage after the 4th moulting.

** Days after the 4th moulting.

*** Days after the 4th moulting.

**** Days after the pupation.

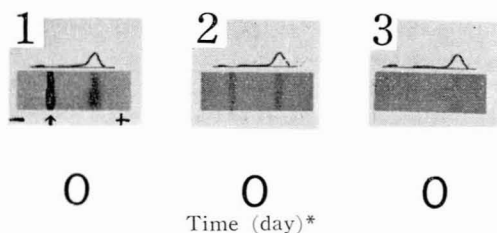


Fig. 4. Electrophoretic patterns of the protein from the soluble fraction of pupal testes of smaller citrus dogs.

* Chemicals were orally administered to newly moulted fifth instar larvae.

The testis was taken from 3 day-old pupae

- (1) Untreated
- (2) Hempa, 4,000 μ g/larva
- (3) Metepa, 4,000 μ g/larva

fraction. It is concluded that the changes in G_1 and G_2 fractions in the hemolymph of treated larvae may be closely connected with chemosterilant-induced structural abnormalities.

In this point, however, further work is necessary to investigate the histopathological, morphological and hormonal variations and also to investigate the relationship between frequency of abnormality of pupae and changes of hemolymph proteins in larvae treated with chemosterilants.

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Summary

The present work was undertaken to investigate the effect of metepa on the hemolymph protein of the last instar larva and pupa of the

smaller citrus dog, *Papilio xuthus* Linne.

In this investigation four main fraction A_1 , A_2 , G_1 , and G_2 were detected from hemolymph of untreated larvae in 4 day-old fifth instar larvae. At 4,000 μ g of metepa and hempa, fraction G_1 decreased remarkably and fraction G_2 decreased slightly. At a dosage of 1,000 μ g of metepa, fraction G_2 was also observed to decrease but the decrease in fraction G_1 was hardly noticeable with hempa. Fraction G_2 in hempa decreased more than the metepa and untreated.

In hemolymph of 1 day-old pupa fraction G_2 clearly decreased following hempa treatment and this was more than that following treatment with metepa.

Only one protein fraction was found in the soluble fraction of the testis in larval and pupal stages. This fraction did not change after treatment with the chemosterilants and the nature of this protein fraction remains unknown.

The A/G ratio of untreated larvae of 4 day-old, fifth instar and pupae was 1.54 and 11.50 to 11.82 respectively. The A/G ratio of treated larvae changed substantially due to the decrease in the G_1 and G_2 fractions.

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