Specific Accumulation of *p*-Hydroxyphenyllactic Acid as a
Metabolite of Tyrosine Metabolism in the Haemolymph
of the Larva of *Bombyx mori* L. (Lepidoptera :
Bombycidae) Intoxicated with MTMC
(*m*-tolyl-*N*-methylcarbamate)

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Carbamate insecticides caused an elevation in the concentration of hydroxyphenyl compounds in the haemolymph of the silkworm larva at treatment with a lower dose. A specific accumulation of HPLA (*p*-hydroxyphenyllactic acid) was evident in the silkworm.

INTRODUCTION

Biologically active chemicals such as insecticides, herbicides and juvenoids, when administered to insects, caused a biochemical alteration in the haemolymph (CORRIGAN and KEARNS, 1958, 1963; MICKS et al, 1960; MANSINGH, 1965; MIYATA and SAITO, 1973; ESSAG et al, 1976; KURODA and GAMO, 1978; SHIGEMATSU, 1978). Several of these authors described cases of application of sublethal or excessive doses of chemicals and the common evidence regarding a drastic change in amino acid content. We were also interested in the effect of carbamate insecticides on the silkworm, especially the change in the content of hydroxyphenyl compounds in the haemolymph.

MATERIALS AND METHODS

Silkworm. A hybrid between Nichi 124 and Shi 124 was reared on mulberry leaves in the usual manner. Six-day-old larvae of the 5th instar were used for specimens in the experiments.

Carbamate insecticides. MTMC (*m*-tolyl-*N*-methylcarbamate), BPMC (2-*sec*-buthylphenyl-*N*-methylcarbamate) and CPMC (2-chlorophenyl-*N*-methylcarbamate) were dissolved in acetone and served for intoxication of larvae. These insecticides were obtained from Hokko Chemical Industry Co., Ltd., Tokyo, Japan.

Standard compounds for TLC of tyrosine metabolites. L-Tyrosine, Dopa (3,4-dihydroxyphenylalanine) and Dopamine (3,4-dihydroxyphenylethylamine) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. HPPA (*p*-hydroxyphenylpyruvic acid) was purchased from Sigma Chemical Co., Missouri, U.S.A. HPLA (*p*-hydroxyphenyllactic acid) was prepared by the following method.

Preparation of HPLA. *p*-Hydroxyphenyllactic acid (III) was prepared from *p*-hydroxyphenylpyruvic acid (II) by reduction with NaBH_4 .

- I X=H, NH_2
 II X=O
 III X=H, OH

The mixture of NaBH_4 , 250 mg, and *p*-hydroxyphenylpyruvic acid, 500 mg, in 10 ml of ethyl alcohol was stirred for 24 hours at room temperature. The end point of reaction was detected with the shift of UV absorption at λ_{max} 334 nm (II) to λ_{max} 292 nm (III) in alkaline solution. The reaction mixture was then dried *in vacuo*, diluted with water, and extracted with ethyl ether.

p-Hydroxyphenyllactic acid (III) was obtained in satisfactory yields after recrystallization from acetone.

Application of carbamate insecticides to larvae. A dose of carbamates lower than LD_{50} was dissolved in acetone and applied topically to larvae. LD_{50} value of each carbamate is as follows: MTMC 788, BPMC 3.47, or CPMC 2051 μg per g body weight of larva on topical application (SUGIYAMA and EMORI, 1970).

Collection of the haemolymph. The haemolymph was collected by cutting the thoracic legs of larvae at various periods after application. The haemolymph from 20 larvae per series was pooled and stored at -20°C until used for the assay.

Determination of hydroxyphenyl compounds as "tyrosine". The hydroxyphenyl compounds in the haemolymph were estimated as "tyrosine" by the expedient method with the UV absorption spectrophotometry after deproteinization in 5% PCA (SHIGEMATSU, 1978). The rough principle for this method was as follows: tyrosine had λ_{max} 279 nm in neutral or acidic solution and showed a red shift to 292 nm with the addition of excessive alkaline. λ_{max} of absorbance at both 279 and 292 nm increased in proportion to the concentration of tyrosine. The simple related formula was induced from λ_{max} and tyrosine concentration. The amount of tyrosine in the haemolymph could be calculated from the values of λ_{max} (nm) obtained by the addition of a definite concentration of tyrosine. It was also possible to estimate *p*-hydroxyphenyllactic acid (HPLA) as one of the metabolites from tyrosine, by means of this expedient method, because HPLA had almost the same λ_{max} and red shift as tyrosine.

TLC analysis for hydroxyphenyl compounds. Hydroxyphenyl compounds in the haemolymph were assayed by the development of TLC plates (Merck, silica gel 60 with concentration zone) with *n*-butylalcohol·acetic acid·water (6 : 2 : 2, v/v) as a solvent system. The plates were exposed to iodine vapor to make each spot visible. Co-chromatography afforded respective R_f values as follows: tyrosine showed 0.57, Dopa 0.48, Dopamine 0.61, HPPA 0.84 and HPLA 0.94. The haemolymph was deproteinized by the addition of 5% PCA, and the metabolites were separated by TLC. Visualized spots were separately scrapped off the plates and eluted with distilled water. The water eluates were assayed for UV absorption spectra. Tyrosine and HPLA were determined by using the red shift of 279 to 292 nm and Dopa and Dopamine were assayed with UV shift of 281 to 302 nm in an alkaline condition. The shift of λ_{max} from 291 to 334 nm was applied to a determination of HPPA.

RESULTS

Effect of carbamate insecticides on the concentration of "tyrosine" in the haemolymph

Three kinds of carbamate insecticides were applied to 6-day-old larvae, average body weight 3.5 g, and the concentration of "tyrosine" was assayed by the expedient method (SHIGEMATSU, 1978). Application doses were 10, 10 and 20 μg per larva for MTMC, BPMC and CPMC, respectively. The results are shown in Fig. 1. All of the insecticides could raise the concentration of "tyrosine" within 24 hours after the application.

Fig. 1. Concentration of "tyrosine" in the haemolymph after application of carbamate insecticides to 6-day-old larvae.

—●—: MTMC 10 $\mu\text{g/larva}$, —○—: CPMC, 20 $\mu\text{g/larva}$, —●—: BPMC, 10 $\mu\text{g/larva}$, —○—: Control

Table 1. CHANGE IN CONCENTRATION OF TYROSINE METABOLITES IN HAEMOLYMPH OF INTOXICATED SILKWORM LARVAE WITH MTMC

		Hours after application ($\mu\text{mole/ml}$)			
		2	4	8	24
Tyrosine	Control	0.541	0.739	0.541	0.900
	Treated	0.648	0.900	0.618	0.873
Dopa	Control	0.188	0.219	0.191	0.148
	Treated	0.188	0.260	0.224	0.204
Dopamine	Control	0.370	0.298	0.317	0.344
	Treated	0.298	0.397	0.410	0.450
HPPA	Control	0.022	0.026	0.026	0.026
	Treated	0.023	0.026	0.023	0.050
HPLA	Control	—*	0.395	0.230	0.197
	Treated	0.439	0.373	0.373	1.825

* undetermined

Change in the content of tyrosine metabolites in the haemolymph after treatment with MTMC

Five μg of MTMC was applied to 6-day-old larvae per body weight to assay for the change of key metabolites of tyrosine metabolism. The metabolites measured were tyrosine, Dopa, Dopamine, HPPA and HPLA after TLC development of the haemolymph, collected at various times after MTMC application. The results obtained are presented in Table 1. Four metabolites other than HPLA did not significantly change in their concentrations during the time-lapse or by the MTMC treatment in the 24 hours following MTMC exposure. In intoxicated larvae, the haemolymph specifically accumulated HPLA after 24 hours of the MTMC application. "Tyrosine" measurable by our expedient method was found to consist of tyrosine and HPLA. The normal haemolymph contained HPLA with about 30% "tyrosine", but the intoxicated haemolymph contained almost 70%.

DISCUSSION

The unique phenomenon presented in this paper is a specific accumulation of HPLA in the haemolymph of silkworm larva intoxicated with MTMC. As a result of this experiment it is believed that carbamate insecticides may accumulate HPLA. In the case of the JH supply, described by SHIGEMATSU (1978), the increase in "tyrosine" concentration should be attributed to the accumulation of HPLA. It is, of course, necessary to verify the specific formation of HPLA in intoxicated larva in subsequent experiments. DDT on the American cockroach (CORRIGAN and KEARNS, 1963), herbicides on the silkworm (KURODA and GAMO, 1978) and juvenoid on the silkworm (SHIGEMATSU, 1978) all caused a lessening in the concentration of tyrosine in the haemolymph within one day after treatment. In the case of silkworm intoxicated with the carbamate insecticides we examined, there was no reduction of tyrosine concentration. Such a discrepancy may be attributed to a difference in action points of chargers in tyrosine metabolism; there are, however, few reports on the problem.

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