Conversion of Carbaryl at the Site of Larval Tissues of the Silkworm, *Bombyx mori* L. (Lepidoptera : Bombycidae)

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(Received December 19, 1971)

¹⁴C-Carbaryl administered orally was quickly distributed in all tissues and converted into a hydroxy-metabolite, 2-hydroxy 1-naphthyl *N*-methylcarbamate. The metabolism of carbaryl was essentially similar at both high and low doses. Two radioactive peaks were observed in the tissues after treatment with the insecticide; the first peak corresponded to the carbaryl initially taken up and the second peak was recognized as its major metabolite. The rate of disappearance of carbaryl and of the formation of its metabolites differed according to the dose of the insecticide. The fat body formed more quickly the hydroxymetabolite than other tissues. In the case of larvae treated with a non-lethal dose, the time of recovery from toxic symptoms coincided with that of the disappearance of carbaryl from the tissues. At this time, the major metabolite reached maximum levels.

INTRODUCTION

Silkworm larvae were rather resistant to carbaryl than other pests belonging to Hemiptera (SUGIYAMA *et al.*, 1971), and with lower dose than LD 50, they convulsed once and rapidly restored to the normal state (KUWANA *et al.*, 1967). Silkworms were characterized to form 2-hydroxy 1-naphthyl *N*-methylcarbamate (2-OH carbamate) from carbaryl as the initial metabolite, which was not seen in toxicated black-tipped leafhoppers (MORIYAMA *et al.*, 1972). Hydroxylation mechanism of carbaryl has already been pointed in several insects (ANDRAWES and DOROUGH, 1967; DOROUGH and CASIDA, 1964; ZAYED *et al.*, 1966; KUHR, 1970) but the silkworm has a peculiar mechanism of hydroxylation. For the clarification of the detoxication mechanism of carbaryl including its hydroxylation in the silkworm, it would be necessary to know the fate of carbaryl at tissue levels, about which we have no knowledge in even other insects. Furthermore, the fate of the hydroxy-metabolite is of interest, in relation to CO₂ output.

MATERIALS AND METHODS

Silkworm larvae

Silkworms, hybrids between strains Nichi 124 and Shi 124, which were 2-day old and 7-day old of the 5th instar larvae, were employed as the test insects.

¹⁴C-Carbaryl

1-Naphthyl *N*-methylcarbamate (carbaryl, Sevin®) labeled with ¹⁴C on the car-

bonyl was purchased from the Radiochemical Centre, Amersham, England. It had a specific activity of $131 \mu\text{Ci}/\text{mg}$ and radiochemical purity of 98%.

Application of ^{14}C -carbaryl to the silkworm

^{14}C -carbaryl dissolved in a small amount of acetone with a 5% emulsifier of Toxi-mal 500 (Sanyo Bō-eki Inc., Tokyo, Japan) was diluted to a definite amount of authentic carbaryl to get the desired specific activities. This was dispersed in distilled water to provide a solution for application. Thirty two μg ($1 \mu\text{Ci}$) of the chemical in $10 \mu\text{l}$ solution was administered orally to each 2-day old 5th instar larvae by a microsyringe having a $1/4$ mm needle. The dosage of the insecticide corresponded to the lethal dose to each larvae. Each 7-day old 5th instar larvae was treated orally with $7.6 \mu\text{g}$ ($1 \mu\text{Ci}$) of carbaryl, which corresponded to the minimum toxic dose ($1.5 \mu\text{g}/\text{g}$ body weight). In these experiments, three larvae were used as a series for the different ages.

Extraction of carbaryl and its metabolites from larvae

The treated larvae with carbaryl were reared on mulberry leaves at 25°C . At fixed intervals after application, the larvae were dissected for removal of five tissues, i.e., gut¹, hemolymph, fat body, posterior division of silk gland and body wall². Hemolymph was collected in test tubes by cutting the dorsal legs and the tissues were rinsed with saline solution (NaCl , 0.85; KCl , 0.05; CaCl_2 MgSO_4 , 0.01%) and frozen in a deep freezer (-20°C). The extraction procedure of the metabolites was similar to the method of ANDRAWES and DOROUGH (1967) but with a reduced scale of extraction. Each of these tissues, kept in a frozen state, was thawed and homogenized with 3 ml of cold acetone and centrifuged for 10 min at 3000 rev/min. The acetone supernatants were combined, evaporated to dryness under reduced pressure, and the residues dissolved in 3 ml of acetonitrile. An equal volume of *n*-hexane was added to the acetonitrile solution and vigorously shaken. This method was repeated three times to eliminate chlorophyll and other contaminants from the acetonitrile layer. To the acetonitrile layer was added 3 ml of distilled water followed by 5 ml of chloroform. After shaking, the chloroform layer was separated and concentrated under reduced pressure. The water layer was mixed with the sediment collected before and the water soluble extracts were obtained by homogenization and centrifugation.

Separation and assay of carbaryl and its metabolites

Thin layer chromatography (tlc) was used for the separation of carbaryl and its metabolites from the chloroform soluble fractions. The plates were silica gel G of 250μ in thickness. The developing solvent was benzene-methanol (9 : 1, v/v). The water soluble substances were subjected to TLC and developed by butanol-acetic acid-water (5 : 4 : 1, v/v/v). Carbaryl and its metabolites developed on TLC plates were detected by a radiothinlayer chromatogram autoscanner (Aloka TRM-1B). The relative ratio of radioactivity of metabolites was calculated by the half width method of scanned autographs. The radioactivity of the mixture of the original extracts before running the chromatography was assayed by a gas-flow counter. These indices were used to deduce total radioactivities of chemical substances in the tissues being fractionable on TLC.

Assay for $^{14}\text{CO}_2$ output from the toxicated larvae

Second day old 5th instar larva treated topically with lethal and non-lethal dose of ^{14}C -carbaryl were placed in a Warburg's vessel. $^{14}\text{CO}_2$ absorbed in KOH was fixed

¹ Gut not containing contents of alimentary canal.

² Body wall containing skin, peripheral fat body and muscle.

as $\text{Ba}^{14}\text{CO}_3$ for the measurement of radioactivity. Besides these, radioactivities of bodies and faeces with gastric juice vomitted were also measured.

RESULTS

Change in toxic symptoms with respective to doses of carbaryl

Change in toxic symptoms shown by silkworm larva treated with $6.78 \mu\text{g}$ of carbaryl per 1 g body weight was outlined in a previous report (SUGIYAMA *et al.*, 1971). The vomitting of gastric juice, knocking down with the ataxia and restoring the feeding activity were orderly shown by poisoned larvae, followed by the death of larva treated with a higher dose or by impaired states with a lower dose. Detailed changes in these symptoms are shown in Fig. 1 as a diagram in relation to the carbaryl dose.

Fig. 1. State of silkworm larvae treated with carbaryl at 4-day old of the 5th instar during the time lapse after application. Normal, quite normal in appearance, feeding and other behaviors; Nsm, normal in appearance but smaller in body size as compared with the controls; Est, weak excitement; Ef, strong excitement; V, vomitting of gastric juice; Sh, shortening of the body; K, loses of coordination ability; M, moribund; D, death.

Separation of carbaryl and its metabolites by tlc chromatography

Carbaryl and two metabolite fractions from chloroform extract of tissues were resolved by tlc. Rf value of authentic carbaryl, detected by iodine vapor was equivalent to 0.58 and it coincided with the Rf value of one of the radioactive substances in the chloroform extracts from treated larvae. Two other radioactive peaks were detected at Rf 0.24 and its origin (Fig. 2). The radioactivity peak from the water soluble extract, named as non-acetone extractable metabolites, was located only at the origin.

Distribution of chloroform and non acetone extractable metabolites in the tissues

Tables 1 and 2 show the amount of chloroform and non acetone extractable metabolites distributed among the five tissues. It is presented as a percentage to the initial dose of carbaryl for 2-day and 7-day old larvae, respectively. Gut contents and the faeces excreted during the given hours were not assayed in these experiments. More

Fig. 2. Radiothinlayer chromatograms of the chloroform extract separated from gut of 2-day old 5th instar larvae at the indicated time lapse after application of ^{14}C -carbaryl.

than 80% of the activity recovered from the tissues were involved in the chloroform soluble metabolites and most of the radioactivity appeared in chloroform soluble fractions of the gut and body wall. The activities of the chloroform fractions in both these tissues fluctuated coincidentally to give two maxima at 1 and 6 hr after application of the lethal carbaryl dose to the 2-day old larva. Other tissues also showed similar patterns of fluctuation and the fat body attained maximum activity faster than the above two tissues. On the contrary, 7-day old larva applied with a non-lethal dose did not distinctly show the two maxima in radioactivity, with the exception of the body wall and posterior division of the silk gland, in which maxima were attained at 1 and 3 hr after application. Percentage of non acetone extractable metabolites was rather high in the 7-day old larva.

Fluctuation of amounts of carbaryl and its metabolites in chloroform extracts of the tissues

Carbaryl taken by the larva was quickly distributed in every tissue, among which the gut and body wall incorporated it very actively. The disappearance of carbaryl and the subsequent appearance of the metabolites were clearly detected in every tissue. Figs. 3 and 4 show the changes in radioactivities of parent carbaryl and two metabolite fractions, i. e., metabolite I, 2-hydroxy 1-naphthyl N-methylcarbamate, and other polar metabolites (metabolite II) for 2-day and 7-day old larvae, respectively. The rate of disappearance of carbaryl and of the formation of 2-OH carbamate in 2-day old larvae treated with a lethal dose was larger than in the 7-day old larvae treated with a non-lethal dose, but the rate of formation of metabolite II was almost the same in both cases. Appearance of 2-OH carbamate in the fat body was faster than

Table 1. RECOVERY OF ^{14}C FOUND IN CHLOROFORM AND WATER SOLUBLE (NON ACETONE SOLUBLE) METABOLITES IN THE TISSUES OF SILKWORM LARVAE AT 2-DAY OLD OF THE 5TH INSTAR ADMINISTERED WITH LETHAL DOSE OF CARBARYL

Tissues tested	Metabolites soluble in	% of radioactivity to the initial dose at indicated hour after intake								
		0.25	0.5	1	2	3	4	6	10	24 hr
Gut ^a	CHCl ₃	8.3	8.8	10.1	4.4	7.3	6.9	13.3	4.4	0.6
	H ₂ O	0.3	0.4	0.3	0.6	0.9	2.4	2.0	1.1	0.8
Hemolymph	CHCl ₃	0.5	0.5	1.6	1.0	1.5	1.3	1.3	— ^c	0.7
	H ₂ O	0.0	0.0	1.0	0.0	0.1	0.4	0.4	—	0.3
Body wall ^b	CHCl ₃	10.9	14.6	18.4	11.1	12.6	13.2	14.0	6.5	0.9
	H ₂ O	0.2	0.3	1.1	0.8	1.1	2.7	2.7	1.8	0.2
Fat body	CHCl ₃	0.4	0.2	0.5	0.5	0.9	0.9	0.5	0.4	0.0
	H ₂ O	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Posterior division of silk gland	CHCl ₃	0.3	0.2	1.0	0.3	0.7	1.7	1.3	0.9	0.2
	H ₂ O	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total recovery in tissues tested	CHCl ₃	20.4	24.3	31.6	17.4	23.0	24.0	30.4	12.2	2.4
	H ₂ O	0.5	0.7	1.5	1.4	2.1	5.5	5.1	2.9	1.3

Silkworm larva, 0.640 g, was administered orally with 32 μg (1 μCi) of ^{14}C -carbaryl.

^a Gut did not contain contents of alimentary canal.

^b Body wall contained skin, poripheral fat body and muscle.

^c These figures were not examined.

Table 2. RECOVERY OF ^{14}C FOUND IN CHLOROFORM AND WATER SOLUBLE (NON ACETONE SOLUBLE) METABOLITES IN THE TISSUES OF SILKWORM LARVAE AT 7-DAY OLD OF THE 5TH INSTAR ADMINISTRED WITH LETHAL DOSE OF CARBARYL

Tissues tested	Metabolites soluble in	% of radioactivity to the initial dose at indicated hour after intake								
		0.25	0.5	1	2	3	4	6	10	24 hr
Gut	CHCl ₃	11.8	10.2	8.1	6.5	4.1	3.8	2.7	1.8	0.6
	H ₂ O	0.8	0.8	1.8	3.4	3.3	3.2	2.7	2.3	1.6
Hemolymph	CHCl ₃	1.8	3.7	2.7	2.1	1.3	0.8	0.5	0.1	0.1
	H ₂ O	0.3	0.5	0.7	1.4	1.8	1.6	2.1	1.5	1.3
Body wall	CHCl ₃	8.2	11.0	13.5	6.8	10.7	6.1	4.4	2.3	1.1
	H ₂ O	0.8	0.8	1.5	1.6	3.1	2.6	2.0	1.4	1.2
Fat body	CHCl ₃	1.3	2.4	2.1	1.4	1.1	0.7	0.4	0.1	0.2
	H ₂ O	0.2	0.3	0.3	0.5	0.6	0.5	0.5	0.4	0.5
Posterior division of silk gland	CHCl ₃	—	2.9	3.6	2.3	3.6	1.9	1.2	1.0	0.7
	H ₂ O	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total recovery in tissues tested	CHCl ₃	23.1	30.2	30.0	19.1	20.8	13.3	9.2	5.3	2.7
	H ₂ O	2.1	2.4	4.3	6.9	8.8	7.9	7.3	5.6	4.6

Silkworm larva, 5.06 g, was administered orally with 7.6 μg (1 μCi) of ^{14}C -Carbaryl.

in the other tissues. In fat body, the amount of 2-OH carbamate was in excess to that of carbaryl. The amount of the polar metabolites was rather significantly large compared with the amount of carbaryl in fat body and posterior division of silk gland, and it was detectable even at 24 hours after application. Time of disappearance

Fig. 3. Change in the amount of carbaryl and its metabolites recovered from chloroform soluble fractions of tissues of 2-day old larvae at indicated hour after application with lethal dose of ¹⁴C-carbaryl.

Larva was administered orally with 32 μg (1 μCi) of ¹⁴C-carbaryl. The amounts of carbaryl and its metabolites were calculated from their radioactivities per individual based on the specific activity of carbaryl initially used for administration. ○—○, carbaryl; ●—●, metabolite I; ⊙—⊙, metabolite II.

Fig. 4. Change in the amount of carbaryl and its metabolites recovered from chloroform soluble fractions of tissues of 7-day old larvae at indicated hour after application with non-lethal dose of ¹⁴C-carbaryl.

Larva was administered orally with 7.6 μg (1 μCi) of ¹⁴C-carbaryl. Other notices are the same as Fig. 3.

of carbaryl from tissues almost coincided with that of restoring to normal activity from toxic symptoms signed at the very initial phase of the action of the insecticide.

Fig. 5. Pattern of ^{14}C -carbaryl conversion in silkworm larva treated with non-lethal dose.
 ○—○, carbaryl; ●—●, metabolite I; ●—●, metabolite II plus water extracts;
 △—△, $^{14}\text{CO}_2$.

Table 3. DISTRIBUTION OF ^{14}C ON SEVERAL FRACTIONS OF THE TREATED LARVA WITH CARBARYL AT 5 HR AFTER INTAKE

Fractions	% of radioactivity to the initial dose of ^{14}C -carbaryl	
	larva treated with non-lethal dose	larva treated with lethal dose
Larval body		
acetone soluble	55.5	60.7
non acetone soluble	10.5	5.1
Faeces with gastric juice vomitted		
acetone soluble	15.8	33.2
non acetone soluble	3.6	0.1
$^{14}\text{CO}_2$ output	13.7	0.8
Total	100.1	99.9

2-day old of the 5th instar larvae were employed.

Output of $^{14}\text{CO}_2$ from toxicated larvae

$^{14}\text{CO}_2$ output was detected only from larva treated with a non-lethal dose of ^{14}C -carbaryl and after the time of the formation of 2-hydroxy 1-naphthyl N-methylcarbamate (MORIYAMA *et al.*, 1972). Table 3 presents the results of the distribution of radioactivities at 5 hr after application for two cases of larvae treated with lethal and non-lethal doses of ^{14}C -carbaryl. Characteristic differences between the two cases of larvae were as follows: $^{14}\text{CO}_2$ output was negligible from larva treated with a lethal dose; acetone soluble materials found in faeces and gastric juice vomitted from lethal dosed larva were twice that of the non-lethal dosed larva; and the amount of non acetone soluble materials were less in the body and excretes of the lethal dosed larva than the non-lethal one. Fig. 5 shows the relationship between intake of carbaryl and formations of metabolites and CO_2 .

DISCUSSION

Carbaryl intaken by silkworm larvae was rapidly converted to 2-hydroxy 1-naphthyl N-methyl carbamate (MORIYAMA *et al.*, 1972) and this conversion was observed in all tissues of the larva. The rate of conversion was largest in the fat body but the amount of formation of 2-OH carbamate was mostly in the gut and body wall. This result will be of concern to the findings that fat body is the most active carbaryl-metabolizing tissue in the blow fly larva (PRICE and KUHR, 1968) and in the cabbage loopers (KUHR, 1970). Since the toxicity of 2-OH carbamate was less than one hundredth of the carbaryl, the formation of this hydroxy-metabolite would contribute to detoxication of the insecticide in the silkworm. In the bollworm, *Heliothis zea*, the major metabolite of carbaryl was suggested to be 1-naphthol formed through the hydrolysis of 1-naphthyl N-hydroxymethylcarbamate, which would be the initial hydroxylated product of carbaryl (ANDRAWES and DOROUGH, 1967). In the silkworm, the formation of 1-naphthol was not examined but the hydroxylation of carbaryl could not be acted, because the output of $^{14}\text{CO}_2$ from carbamate was not detected unless 2-OH carbamate was formed. The hydroxy-metabolite of carbaryl would be easily hydrolyzed. The mechanism of hydrolyzation of 2-OH carbamate is under examination from the viewpoint of detoxication of carbamates in insects. In this respect, more polar metabolites, such as metabolite II and non acetone soluble metabolites, would possibly be subsequent products of 2-OH carbamate. In larva treated with a lethal dose of carbaryl, the little $^{14}\text{CO}_2$ output and less formation of non acetone soluble materials lead us to suspect that heavily toxicated larva may inhibit further transfer of 2-OH carbamate.

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