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METABOLISM OF CARBAMATE DRUGS—II: DEGRADATION OF 1-NAPHTHYL-N-METHYL CARBAMATE (SEVIN) IN ADULT LARVA OF THE COTTON LEAF WORM (*PRODENIA LITURA* F.)

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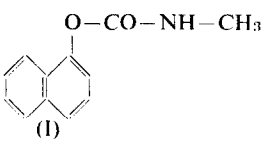
Abstract—The metabolic fate of Sevin has been investigated in the adult larva of *Prodenia litura* F., using ^{14}C-Sevin, labelled at two different sites. From the topically applied dose, 32–37 per cent was recovered as ^{14}C-metabolites in the excreta and the expired air after 20 hr. The insecticide is mainly detoxified by a non-hydrolytic mechanism, probably involving hydroxylation of the aromatic ring. The minor hydrolytic pathway (about 14 per cent) involves hydrolysis of the ester bond to give l-naphthol and N-methyl carbamic acid. The latter decarboxylates to give methylamine which undergoes a process of oxidative demethylation to produce ^{14}C-formate and $^{14}CO_2$. A scheme has been suggested for the oxidative pathway of the methyl group of Sevin.

AMONG carbamates, recently discovered as a group of compounds possessing insecticidal activity,[1] 1-naphthyl-N-methyl carbamate (I) (Sevin) was the first to be discovered that found extensive use in the field of crop protection. This insecticide possesses a broad spectrum of activity and a low order of mammalian toxicity.[2]



(I)

It is a contact insecticide with long residual properties[3] and was found to be effective in controlling cotton pests, e.g. pink boll worm *Pectinophora gossypiella*, and boll weevil *Anthonomus grandis*.[4] It has also found application in controlling the cotton leaf worm (*Prodenia litura* F.).

The present investigation is concerned with the study of the metabolic fate of Sevin in the adult larva of the cotton leaf worm. For this study two, types of radioactive insecticide in which the C=O group (Sevin I) or the methyl group (Sevin II) is ^{14}C-labelled, have been used.

MATERIALS

^{14}C-*Sevin*

(a) *Sevin I.* Sevin in which the carbamate carbon atom is ^{14}C-labelled* has been

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diluted with non-active pure Sevin (m.p. 142°) so that the resulting radioactive insecticide possessed a sp. act. of $1{\cdot}7 \times 10^5$ counts/min per mg.

(b) *Sevin II.* Sevin in which the methyl-C is ^{14}C-labelled was prepared as described in a former paper,[19] after using the Lambrech[5] method.

Prodenia larvae

For the distribution and metabolism experiments, laboratory reared larvae (fifth to sixth instars) of *Prodenia litura* F. were used after 3 hr starvation. For the *in vitro* experiments 30 per cent total homogenate of the adult larva was used. The homogenate was prepared by homogenizing the larvae in phosphate buffer, followed by filtration through cheese cloth.

METHODS

The radioactivity present in aqueous media and in biological samples was determined (after being dried over P_2O_3) according to the procedure described by Aronoff,[6] using Van Slyke–Folch reagent.[7] All radioactivity measurements were determined as Ba $^{14}CO_3$ in an end-window counter under uniform geometrical conditions, and corrected for background and self absorption. For the determination and characterization of the ^{14}C-metabolites, radio–paper chromatography has been used, using a Frieske and Hoepfner radioscanner. Metabolites with a naphthol rest were made visible by spraying with *p*-nitrobenzene diazonium fluoroborate reagent.[8]

Distribution

The distribution of Sevin among the different organs in the adult larva of the cotton leaf worm was studied following topical application. For this study larvae of almost the same size and weight were used. For each g insect 100 μg of Sevin I (17,000 counts/min) in 20 μl acetone were applied on the dorsal side of the insect. After the elapse of different periods of time, the unabsorbed insecticide was removed by acetone. A sample of the haemolymph was collected and the insect was then rapidly dissected under light petroleum ether (b.p. below 40°). Haemolymph, gut and fat were freshly weighed and measured for their radioactivity.

In vivo *experiments*

The radioactivive insecticide, dissolved in acetone was applied topically (100 μg/g insect). The larvae were left for 20 hr in a beaker containing a little sawdust to ensure healthy conditions for the larvae. After removing the larvae, the beaker contents were extracted five times with ether; using 20 ml for each extraction. The residue was then extracted with water in a similar manner. Both ether and water extracts were measured for their ^{14}C-activity and then concentrated. Samples of the concentrated extracts were analysed for possible metabolites by paper chromatography, using Schleicher & Schüll paper 2043 b.

^{14}C-*activity in the expired air*

For the determination of the ^{14}C-activity in the expired air, the respiratory $^{14}CO_2$, evolved during 20 hr, was trapped by 1 N sodium hydroxide solution,[9] and determined as Ba $^{14}CO_3$.

^{14}C-formate in the excreta

For the identification of the ^{14}C-formate resulting from Sevin II, the aqueous extract of the excreta was concentrated under vacuum. The inverse isotope dilution technique described by Hassan and Zayed[10] was then followed. The ^{14}C-formate in the excreta was determined in terms of specific activity. For further characterization the isolated formate was chromatographed in three solvent systems.

In vitro *experiments*

^{14}C-Sevin (I or II) was incubated with the larval homogenate at 30° in a closed system for 4 hr. The reaction mixture had the following composition: 6 ml aqueous solution of Sevin (12 μg/ml); 2 ml 30% larval homogenate in phosphate buffer (0·2 M, pH 7·0). The evolved $^{14}CO_2$ from Sevin I and II was trapped in a central well containing 10% sodium hydroxide solution. For trapping basic volatile ^{14}C-metabolites, 12 N sulphuric acid was used in a separate experiment. To avoid retention of the basic volatile compounds, saturated sodium carbonate solution (1 ml) was added at the end of the reaction.

RESULTS

Distribution

The distribution of ^{14}C-activity among haemolymph, gut and fat, after topical application of Sevin I has been studied for 5 hr. The results are shown in Table 1.

TABLE 1. DISTRIBUTION OF ^{14}C-ACTIVITY IN *Prodenia* LARVA AFTER TOPICAL APPLICATION OF 100 μg OF SEVIN I/g INSECT

Organ	^{14}C-Activity counts/min per g fresh wt.* after (hr)			
	0·5	1	3	5
Haemolymph	900	2250	4700	5200
Gut	500	1600	2600	2700
Fat	670	1750	3400	6400
Total insect	520	1600	3200	4300

* Values are mean of eight determinations.

From the applied dose (17,000 counts/min per g insect) about 25 per cent of the ^{14}C-activity was absorbed in 5 hr. The ^{14}C-activity in the fat showed a definite, progressive increase with time.

In vivo *studies*

Sevin I. For this investigation a pool of 10 g insects has been used. After 20 hr, 39·3 per cent (67,000 counts/min) of the applied dose could be recovered in the combined ether extracts of the excreta. From several chromatograms, it has been estimated that Sevin contributed to about 20 per cent of the ether extractable ^{14}C-activity; the rest being due to a metabolite. Its R_f-values in three solvent systems are listed in Table 2. The combined aqueous extracts, on the other hand, proved to contain only a small percentage of radioactivity (0·8 per cent of the applied dose). In a separate experiment, it was shown that exhaustive ether extraction of the excreta, reduced the

^{14}C-activity in the aqueous extracts to 0·2 per cent. This activity was found to be due to the same ether-soluble metabolite.

The respiratory $^{14}CO_2$, collected during 20 hr accounted for 4·94 per cent of the initially applied dose (8400 counts/min in the $Ba^{14}CO_3$ precipitate). ^{14}C-activity remaining in the larvae was found not to exceed 1 per cent.

TABLE 2. R_f-VALUES OF ^{14}C-METABOLITES OF SEVIN

System	Sevin	^{14}C-formate	R_f-values (*)		
			Ether sol. metabolite		Water sol. metabolite
			Sevin I	Sevin II	Sevin II
Methanol:water (8:2)	0·95	0·69	0·77	0·75	0·69
Methanol:water (1:1)	0·78	—	0·61	0·61	—
Methanol:water (4:6)	0·76	—	0·64	0·63	—
Methanol: acetic:water (4:1:5)	0·83	0·77	—	—	0·77
n-Butanol sat. 1·5N ammonia	—	0·11	—	—	0·11

* Reproducible R_f-values were obtained with freshly prepared systems.

Analysis of sawdust for its ^{14}C-activity showed that it retained 53 per cent of the applied radiodose; thus demonstrating an excellent recovery of the radioactivity.

Sevin II. A pool of 10 g insect, was treated with Sevin II (100,000 counts/min). The total ^{14}C-activity recovered in the excreta and the expired air accounted for about 41 per cent of the applied dose. Sevin—determined as previously described—contributed to 9 per cent of the initial dose. Of the administered dose, 2·5 per cent was present in the aqueous extracts. $^{14}CO_2$ eliminated in the expired air during 20 hr was found to account for 7·2 per cent of the total ^{14}C-metabolites (2·3 per cent of the applied dose). This gives a mean value of 0·057 μmoles/hr per 100 g insect for the "apparent rate" of oxidative demethylation of the insecticide.

The ether extract proved to contain—in addition to the unchanged Sevin—a main metabolite, which is identical with the ether soluble ^{14}C-metabolite obtained from Sevin I (Table 2).

Paper chromatographic analysis of the water-soluble metabolite(s) gave mainly one spot, whose R_f-value was similar to that of ^{14}C-formate which was run alongside as reference (Table 2). The presence of ^{14}C-formate in the aqueous extracts, has been proved by the inverse isotope dilution technique. Its amount contributed to 4·5 per cent of the total metabolites; or 1·43 per cent of the initially applied dose.

Alkaline degradation of the ether soluble metabolite. When the ether soluble components were subjected to alkaline hydrolysis (2 N sodium hydroxide solution for 20 min), followed by acidification, the ^{14}C-activity disappeared completely. The ether extract of the acidified reaction mixture was used for investigating the phenolic

constituents by paper chromatography. In addition to a small amount of 1-naphthol another phenolic substance could be detected (Table 3).

Metabolic fate of 1-naphthol. The metabolic fate of 1-naphthol in *Prodenia* larvae has been studied in the same manner as Sevin. The excreta was investigated for possible metabolites of 1-naphthol. In addition to unchanged naphthol, a modified phenol was also detected (Table 3). Alkaline hydrolysis of the excreta gave only 1-naphthol.

TABLE 3. R_f-VALUES OF 1-NAPHTHOL METABOLITE(S) *in vivo* AND PHENOLIC COMPONENTS OBTAINED BY ALKALINE HYDROLYSIS OF THE ETHER SOLUBLE METABOLITE OF SEVIN

System	1-Naphthol	Naphthol metabolite		Hydrolysed ether-soluble components
		Before hydrolysis	After hydrolysis	
Methanol:water (8:2)	0·92	0·20 0·91	0·92	0·76 0·92
Methanol:water (1:1)	0·80	0·32 0·80	0·79	0·74 (tailing)
Methanol:water: acetic acid (4:5:1)	0·79	—	0·81	0·68 0·78

In vitro *experiments*

The degradation of ^{14}C-Sevin to $^{14}CO_2$ by larval homogenate has been studied. When Sevin I was used, about 800 counts/min (equivalent to 4·7 μg insecticide) were recovered in the $Ba^{14}CO_3$ precipitate (mean of 5 determinations). On the other hand, the amount of $^{14}CO_2$ recovered from Sevin II was much smaller and accounted only for less than 0·3 per cent of the initial dose.

The radioactivity trapped in sulphuric acid, when Sevin II was used, was negligible (about 1 per cent of the initial dose).

DISCUSSION

It has been demonstrated that the effectiveness of Sevin against a wide variety of insects is due to its anticholinesterase properties.[11] Sevin has been considered economically effective against the cotton leaf worm[12] and the pink boll worm.[4] The present investigation has been stimulated after an observation that Sevin possesses a lower toxicity than Dipterex against *Prodenia* larvae. From distribution studies, it was found that about 25 per cent of the applied dose of Sevin was absorbed during 5 hr by the adult *Prodenia* larvae; indicating a fair absorption rate of the insecticide through the skin. This rate is much smaller than that of organophosphorus insecticides, e.g. Dipterex.[13] Though radioactivity in gut and haemolymph increased only slightly after 3 hr from topical application, that in the fat increased significantly (Table 1).

The absence of cholinesterase from the larva[14] and the low absorption rate of Sevin may account for the low toxicity of the insecticide to *Prodenia* larvae. Rapid rates of detoxification may be as well a contributing factor for the low toxicity. From metabolism studies, it was shown that at least 32–37 per cent of the applied dose was

metabolized in 20 hr. This percentage does not represent the true rate of detoxification; since it was impossible to relate the amount of metabolites to the "absorbed dose". Again, the ^{14}C-activity retained by the sawdust may be due to unchanged insecticide and/or ^{14}C-metabolites. The whole phenomenon suggests a fairly rapid rate of detoxification in *Prodenia* larvae.

The *in vivo* metabolism studies showed that the main metabolite of Sevin I was similar to that obtained from Sevin II. The radioactive spots of the main metabolite gave greenish colour characteristic for phenols; after spraying the chromatogram with the fluoroborate reagent. This indicates the presence of a phenolic rest in the metabolite.

These findings prove the presence of the skeleton (C—O—C(=O)—N—C); thus demonstrating a major non-hydrolytic pathway for the detoxification of Sevin in *Prodenia* larva. This metabolite was found to be relatively stable in acid medium but could be easily degraded when boiled with dilute alkali to give a modified naphthol. Though it is believed that the ether-soluble metabolite is a single component; yet the probability that it constitutes more than one substance having close structures cannot be precluded.

Studies on the metabolism of Sevin in insects has been recently carried out. It has been assumed that the metabolism of N-methylcarbamates, generally involves initial esterase attack followed by degradation of the hydrolysed fragments.[15, 16] Later work indicates the presence of a nonhydrolytic route for the degradation of Sevin, which represents a main metabolic pathway in insects as in mammals.[17–19] Liver homogenates and cockroaches treated with ^{14}C-Sevin were reported to produce five metabolites

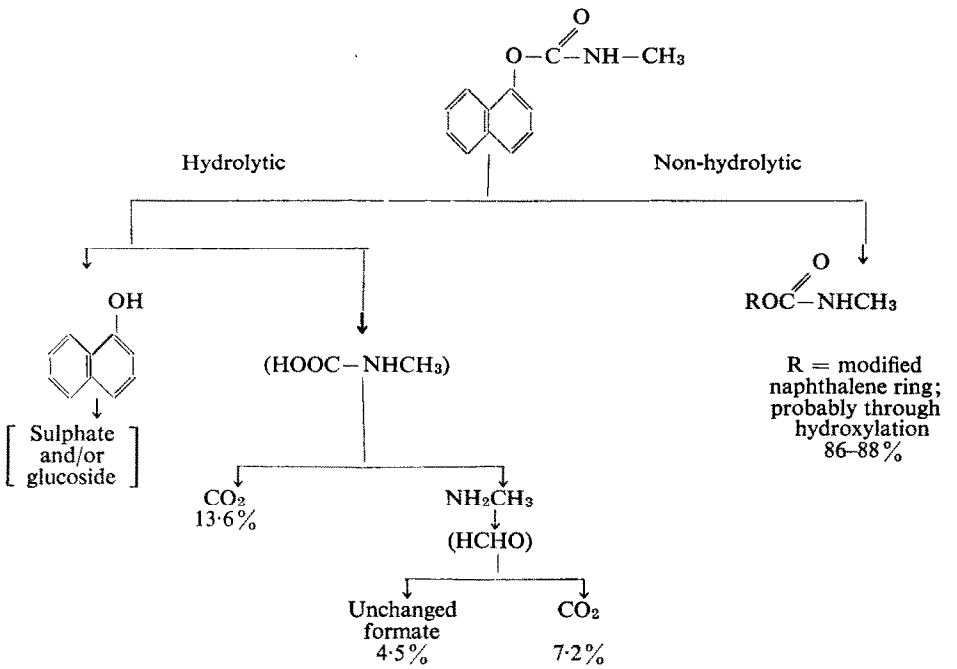
possessing the skeleton C—O—C(=O)—N—C.[17]

Concerning the nature of the major metabolite, it is believed that the aromatic ring has been modified by a hydroxylation process. This receives support from the finding that aromatic rings can be detoxified in insects through hydroxylation.[20]

Moreover, Dorough and Casida[18] believed that hydroxylation rather than hydrolysis constitutes the major detoxification mechanism of Sevin in insects. It is rather improbable that the methyl group of the ether-soluble metabolite has undergone any modification. In favour of this assumption, is the work of Dorough and Casida,[18] who reported that none of the ^{14}C-metabolites of Sevin – having a hydroxyl group in the naphthol rest suffers any change of the methyl group. Coupling of the main metabolite with a reducing agent has been excluded by reduction tests. It is also believed that this metabolite is not conjugated with a detoxifying agent (e.g. excreted as ethereal sulphate). This receives support from the observation that the major metabolite is ether soluble. In this connection it is worth mentioning that Dorough and Casida[18] reported that the pathway of detoxification of Sevin in insects does not include coupling with glucose, glucuronic acid or sulphate.

Apart from the non-hydrolytic pathway, a minor hydrolytic mechanism contributes to the detoxification of Sevin. This consists of hydrolysis of the ester bond by the action of esterases. The liberated N-methyl carbamic acid undergoes spontaneous decarboxylation to give methylamine (Fig. 1). This is indicated by the elimination of $^{14}CO_2$ (from Sevin I) in the expired air. The process of decomposition probably takes

place uncatalysed by enzyme systems. The esterase action has been also found to take place *in vitro*. Evidence for the possible formation of $NH_2{}^{14}CH_3$ from Sevin II, has been gained from the *in vitro* studies, where a trace of radioactivity could be trapped in sulphuric acid.



* Percentages are related to total ^{14}C-metabolites.

FIG. 1. Possible metabolic pathways of Sevin in *Prodenia litura* F.*

It is believed that 1-naphthol, liberated on hydrolysis of the ester bond does not undergo ring modification; but excreted, at least partly, in a conjugated form. Evidence for this belief is gained from the preliminary investigations on the metabolic fate of 1-naphthol.

The elimination of radioactive carbon dioxide in the expired air after topical application of Sevin II proves the oxidative degradation of the methyl group. It is believed that the primary aliphatic amine—rather than Sevin itself—undergoes this degradation process. The elimination of $^{14}CO_2$ from Sevin II does not represent the true demethylation rate; since ^{14}C-formate has been isolated and identified as a second metabolite.

The $^{14}CO_2$ elimination from Sevin II is believed to be accomplished by a multi-enzyme system. The latter consists presumably of an amine oxidase, aldehyde oxidase and catalase and the whole reaction chain proceeds as follows:

$$\text{methylamine (or Sevin)} \xrightarrow{\text{amine oxidase}} \text{formaldehyde} \quad (1)$$

$$\text{formaldehyde} \xrightarrow[\text{aldehyde oxidase}]{\text{catalase}} \text{formate} \quad (2)$$

$$\text{formate} \xrightarrow{\text{catalase}} \text{carbon dioxide.} \quad (3)$$

Aldehyde oxidase and catalase were recently reported to be present in the larval haemolymph.[9] The identification of ^{14}C-formate—as a metabolite of Sevin—supports the suggested pathway; which is believed to occur also *in vitro*. Scheme 1 represents the possible metabolic pathways of Sevin in *Prodenia litura* larvae.

The oxidative degradation of the methyl group in *Prodenia* larvae has been first demonstrated by Hassan *et al.*[9] from metabolism studies with organophosphorus esters, e.g. Dipterex. Though ^{14}C-formate is eliminated as a degradation product of Sevin, it could not be isolated as a metabolic product of the methyl groups of the Dipterex molecule. Two factors may contribute to these findings: (1) The methyl groups of Dipterex are first liberated as mathanol; a major part of which follows a non-oxidative pathway. On the other hand, formaldehyde constitutes the first degradation product of the methyl group of Sevin. (2) The enzymatic systems involved in the transformation of formaldehyde to carbon dioxide might be partially inhibited by Sevin.

As mentioned before, Sevin has been found to be effective in controlling the pink boll worm (*Pectinofera gossypiella*). Investigation of the *in vivo* metabolism of the insecticide by *Pectinofera* larvae, showed that the metabolites were the same as those obtained from *Prodenia* larvae.

The larvae of the pink boll worm were found to lack cholinesterase as determined by the method of Hestrin.[21] In this respect this insect similates the larvae of the cotton leaf worm.[14]

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