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METABOLISM OF CARBAMATE DRUGS—I

METABOLISM OF 1-NAPHTHYL-N-METHYL CARBAMATE (SEVIN) IN THE RAT

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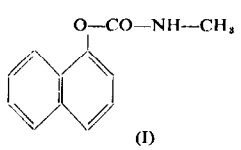
Abstract—The metabolic fate of Sevin in the rat has been investigated *in vivo* and *in vitro*, using ^{14}C-Sevin labelled at two different sites. Following a single injection (i.p.) of radioactive Sevin, 75–80 per cent of the ^{14}C-activity was recovered—after 48 hr—in the expired air and in the urine. Hydrolysis of the ester bond liberates N-methyl carbamic acid which decomposes spontaneously to methylamine and carbon dioxide. The latter contributed 43·5 per cent of the recovered radioactivity. The methylamine moiety of the insecticide was oxidatively demethylated to $^{14}CO_2$ which was eliminated in the expired air, and C^{14}-formate which was excreted in the urine. The radioformate accounted for 9·2 per cent of the administered dose. Demethylation of the intact Sevin molecule does not occur *in vivo*. A nonhydrolytic pathway involving modification of the methyl group gave a metabolite (56·5 per cent of the recovered radioactivity) which was excreted in the urine in a conjugated form, possibly as glucuronide. A formula has been suggested for this metabolite, which still retains the skeleton of the Sevin molecule. The contribution of both mechanisms (hydrolytic and nonhydrolytic) to the detoxification of the insecticide has been discussed.

THE CARBAMATE insecticides are becoming increasingly important in insect control. The insecticide Sevin [1-naphthyl-N-methyl carbamate (I)] proved to be effective



(I)

against a variety of insects attacking fruit trees and bean and cotton crops. It derives its toxic effects from being a potent cholinesterase inhibitor.[1] Of particular interest, is that the anticholinesterase activity of the insecticide is greater in insects than in mammals; and in general its toxicity for mammals is of low order.[2]

The metabolism of Sevin has been recently investigated in mammals and insects; and the mechanism of biological degradation has been claimed to involve modification of both the ring and the methyl group.[3] Whitehurst *et al.*[4] reported that the insecticide appears as 1-naphthol or Sevin or both in the urine of cows. Eldefrawi and Hoskins[5] believed that the first step in the degradation of Sevin involves the action of a carbamate esterase. Since the rate of degradation of the insecticide *in vivo* proceeds more rapidly than can be accounted for by the hydrolytic action of esterases, it is believed that another degradation mechanism(s) is involved. In fact, Dorough *et al.*[3] reported

that rat liver microsomes degrade Sevin—by a nonhydrolytic mechanism—to five metabolites.

Still, the metabolic pathways of Sevin—hydrolytic and nonhydrolytic—remain to be clarified. Also, the fate of the substituted amine moiety of the insecticide is not yet known. In the present work, the metabolic fate of Sevin in the rat has been investigated, in an attempt to elucidate the mechanism of detoxification of the insecticide and to identify its metabolites. For this purpose, ^{14}C-Sevin, labelled at two different sites, has been used.

MATERIALS AND METHODS

^{14}C-*Sevin*

0·5 ml 1-Naphthyl-N-methyl (carbamate-^{14}C)* (Sevin I) was diluted with non-radioactive Sevin (m.p. 142°). The resulting mixture possessed a specific activity of $1{\cdot}7 \times 10^5$ counts/min per/mg.

1-Naphthyl-N-(methyl-C^{14}) carbamate (Sevin II) has been prepared from 1-naphtyl chloroformate, which was prepared from 1-naphthol and phosgene after the method of Lambrech[6] (b.p.[10] 128–136°; lit. b.p.[2] 96–100°). To a mixture of 0·44 ml of the chloroformate, about 0·42 g of ^{14}C-methylamine hydrochloride,† and 1 ml water; 1 g of crystalline sodium acetate was added and the reaction flask was stoppered and shaken for 6 hr. The reaction mixture was allowed to stand overnight and the solid substance was centrifuged, dried and crystallized twice from carbon tetrachloride, m.p. 141–142°; sp. act. 1×10^5 counts/min per mg.

In vivo *experiments*

For this investigation, albino rats of both sexes, weighing 100–120 g were used. Radioactive Sevin was dissolved in the least amount of ethanol (0·5 ml/30 mg) and injected intraperitoneally (30 mg/kg). For the collection of respiratory $^{14}CO_2$, metabolic cages were used.[7] The $C^{14}O_2$ was trapped by 1 N sodium hydroxide solution, and determined as $Ba^{14}CO_3$. The urine was collected in a receiver containing 0·3 ml saturated sodium carbonate solution to avoid any loss of ^{14}C-activity. ^{14}C-activity in aqueous media and different organs was determined (after the samples were dried over P_2O_5) as described by Aronoff,[8] using Van Slyke–Folch oxidizing reagent.[9]

All radioactivity measurements were carried out in an end-window counter, and corrected for background and self-absorption.

^{14}C-metabolites in the urine and homogenate extracts were investigated by paper chromatography. The ascending chromatographic technique was adopted, using Schleicher & Schüll paper 2043 b. The chromatograms were assayed radiometrically using a Frieske & Höpfner radioscanner. Phenolic constituents were identified by spraying the chromatograms with *p*-nitrobenzene diazonium fluoroborate reagent.[10] Phenolic components in urine were determined colorimetrically, using 4-amino-antipyrin.[11] For isolation and determination of ^{14}C-formate in the urine, the inverse isotope dilution technique was used. The procedure is that essentially described by Hassan and Zayed.[12] Characterization of the isolated radioformate was achieved by paper chromatography in two different solvent systems.

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† ^{14}C-Methylamine hydrochloride (0·5 mc; Amersham, Buckinghamshire, England) was diluted with 410 mg non-labelled methylamine hydrochloride.

In vitro *experiments with tissue homogenates*

Homogenates from different organs were incubated at 37° for 3 hr, in a buffered medium with an aqueous solution of Sevin. Owing to the low solubility of the insecticide in water, the substrate concentration was low (10–12 μg/ml). The $^{14}CO_2$ which evolved during the incubation period was trapped in 10% sodium hydroxide solution contained in a central well.

In a series of experiments with Sevin II, the reaction mixture was incubated in the presence of 10 mM (final concentration) semicarbazide solution. At the end of the reaction, the contents were centrifuged, and the supernatant was extracted three times with chloroform. The combined chloroform extracts were evaporated to dryness. Carrier paraformaldehyde in water (2 mg/flask) was then added, and the pH of the solution adjusted to about 4. Aqueous dimethone solution was added and the whole mixture was left over-night at 2°. The crystallized product was filtered and counted in much the same way as $Ba^{14}CO_3$.[13]

Monoamine oxidase activity

Liver MAO activity has been assayed in presence of Sevin, using tyramine as a substrate. The enzyme activity was assumed to be proportional to the amount of liberated ammonia, which was determined according to the procedure of Seligson.[14] The reaction mixture was incubated at 37° and had the following composition: 1 ml phosphate buffer, pH 7·0, 0·2 M; 1 ml 10% liver homogenate in isotonic KCl; 6 ml Sevin solution (12 μg/ml; 1 ml tyramine solution (90 mM)).

RESULTS

^{14}C-activity in the expired air

Figure 1 illustrates the cumulative elimination of $^{14}CO_2$ in the expired air during 24 hr. Following the administration of Sevin I, the production of $^{14}CO_2$ starts at once, and within 6 hr the major part (about 87 per cent) has been eliminated. This corresponds to about 26 per cent of the injected dose and gives a mean value of 0·65 μmoles/hr per 100 g rat for the rate of hydrolysis of the drug at the naphthyl-carbamate bond. With Sevin II, the elimination of $^{14}CO_2$ proceeds at a slower rate, and after 6 hr, about

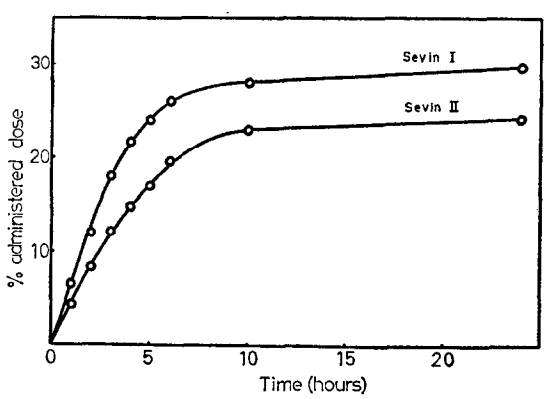


FIG. 1. Elimination of ^{14}C-activity in the expired air ($^{14}CO_2$) after the administration of a single dose of radioactive Sevin (30 mg/kg). Data are mean of three experiments.

19·8 per cent of the radioactivity of the administered radiosevin could be recovered in the expired air. This gives a mean value of 0·49 μmoles/hr per 100 g rat for the "apparent" rate of demethylation of Sevin.

The rate of elimination of respiratory $^{14}CO_2$—from Sevin I and II—follows a typical 2-phased curve. During the first 6 hr, a steady state is attained and the process proceeds with almost constant velocity. This phase represents the maximum rate of hydrolysis of the ester bond (Sevin I), as well as the maximum rate of "apparent" oxidative demethylation (Sevin II). The second phase is characterized by a decreased rate of elimination of ^{14}C-activity.

^{14}C-activity in the urine

The percentage of ^{14}C-activity recovered in the urine, is shown in Table 1. The ^{14}C-

TABLE 1. C^{14}-ACTIVITY ELIMINATED IN THE EXPIRED AIR AND IN THE URINE

Sevin	Percentage of ^{14}C-activity (100% = administered dose)				No. of ^{14}C metabolites in urine
	In the expired air (24 hr)	(48 hr)	In the urine (24 hr)	(48 hr)	
I	29·8	32·7	34·2	42·5	1
II	24·3	27·0	43·0	52·6	2

Data are mean of three experiments.

activity recovered from Sevin II exceeded that recovered from Sevin I. Paper chromatographic analysis revealed that urine from both isotopes contained a common ^{14}C-compound which is certainly not Sevin (Table 2). Treatment of the paper chromatograms with *p*-nitrobenzene diazonium fluoroborate reagent, gave greenish spots through partial hydrolysis) at about the positions corresponding to the R_f values of the main metabolite. These findings indicate clearly that this major metabolite possesses the skeleton C—O—C(O)—N—C. It is the only ^{14}C-metabolite obtained in urine from Sevin I, and contributes 77–84 per cent of the radioactivity recovered in the 24-hr urine from Sevin II. Ether extraction of the acidified urine did not transfer more than 7 per cent of this metabolite into the organic layer. When chromatograms of urine were sprayed with 2 N HCl, heated 5 min under a germicidal lamp, and then sprayed with ammoniacal silver nitrate solution and heated, brownish spots appeared at about the positions corresponding to the R_f values of the main metabolite. The spots did not form, if treatment with HCl had been omitted.

^{14}C-formate in the urine

By the inverse isotope dilution technique, ^{14}C-formate was found to contribute 16–23 per cent of the ^{14}C-activity in the 24-hr urine of rats treated with Sevin II. This corresponds to about 8·4 per cent of the injected dose. During the next 24 hr, only about $\frac{1}{10}$ of this amount could be recovered as ^{14}C-formate. The isolated radioformate was further characterized by paper chromatography in: *n*-butanol saturated with concentrated ammonium hydroxide (R_f 0·09); and system D (R_f 0·77) (cf. Table 2). In this connection, it was found that ^{14}C-formate could not be adequately separated (by paper chromatography) from the main ^{14}C-metabolite in the urine. For example,

TABLE 2. R_f VALUES OF SEVIN METABOLITES

System*	R_f†						
	Sevin	1-Naphthol	Alkaline boiled urine from Sevin I and II	Urine from Sevin I	Urine‡ from Sevin II	*In vitro* metabolites of Sevin I	*In vitro* metabolites of Sevin II
A	0·95	0·92	0·92	0·80	0·80	0·49, 0·95	0·47, 0·94
B	0·78	0·80	0·80	0·66	0·66	0·27, 0·79	0·27, 0·78
C	Front	0	0	0	0	—	—
D	0·83	0·79	0·80	0·83	0·82	—	—

* System A: methanol–water (8:2); System B: methanol–water (1:1); System C: benzene saturated with 1:1 aqueous methanol; System D: methanol–water–acetic acid (4:5:1).

† Reproducible R_f values were obtained only with freshly prepared systems; development time 6–8 hr. R_f values in columns 3 and 4, were determined by spraying the chromatograms with *p*-nitrobenzene diazonium fluoroborate reagent. R_f values in columns 2, 5, 6, 7, 8 were determined radiometrically.

‡ After removing ^{14}C-formate by steam-distillation.

the main metabolite possesses R_f 0·0 in *n*-butanol saturated with concentrated ammonium hydroxide, and R_f 0·83 in system D.

Degradation of urine components

When urine of rats treated with Sevin I was boiled for 20 min with 10% sodium hydroxide solution—followed by acidification to decompose the formed $Na_2{}^{14}CO_3$—it lost its ^{14}C-activity completely. Under similar conditions, the urine from Sevin II lost about 80 per cent of its radioactivity. When urine from Sevin I was subjected to acid hydrolysis (10 N HCl) for 40 min, it lost over 90 per cent of its ^{14}C-activity. On the other hand, urine from Sevin II retained about 80 per cent of its radioactivity, when treated in the same manner.

For identification purposes, a part of the alkaline boiled urine was acidified and extracted with chloroform. The organic layer was concentrated and chromatographed. Table 2 shows that phenolic compounds with R_f values similar to those of 1-naphthol, were the only phenolic substances to be detected. When similarly treated, normal urine showed no detectable spots.

In a series of experiments to determine the amount of phenolic constituents eliminated in urine, 24 hr after injecting Sevin, a part of the urine was boiled for 40 min with 10 N HCl to effect the hydrolysis of conjugated naphthols and similar components. Using 4-amino antipyrin, colorimetric measurement of the isoamyl alcohol layer (solvent used for colour extraction) gave a mean value of 960 μg of phenolic compounds in the 24-hr urine. This value was corrected for the phenols normally present in urine (70–120 μg).

Metabolism studies in vitro

Table 3 shows the activity of different organs in degrading Sevin. In liver, kidney, spleen and blood, the hydrolysis of the ester bond produces more or less equal amounts of $^{14}CO_2$, during 3 hr. Blood contains the highest activity of the oxidative-demethylating system, whereas the brain affords the lowest activity. When paper chromatographed, the chloroform extract of the reaction mixture containing liver homogenate proved to contain mainly Sevin and a small amount (about 2 per cent) of a ^{14}C-metabolite produced from Sevin I and II, (Table 2).

In a trial to demonstrate the formation of $NH_2{}^{14}CH_3$, liver homogenate was incubated with Sevin II in a buffered medium (pH 6·5). At the end of the reaction, saturated potassium carbonate solution was added to drive off the alkyl amine (and ammonia resulting from the degradation of methyl amine) which was trapped in sulphuric acid. The latter proved to contain only a negligible amount of radioactivity; indicating that methylamine becomes involved in a reaction at a rate almost equal to that of its formation.

From the experiments designed for trapping ^{14}C-formaldehyde, about 400 counts/min (equivalent to 4 μg insecticide) could be recovered in the dimethone precipitate.

The data presented in Table 4 show that liver monoamine-oxidase is inhibited in presence of low concentrations of Sevin (about 45 μM). The addition of tyramine protects the enzyme against inhibition.

DISCUSSION

When injected into the rat, ^{14}C-Sevin is fairly rapidly metabolized and the metabolic products are excreted via the lungs and kidneys. These constitute the main channels

TABLE 3. CO_2 PRODUCTION FROM SEVIN BY TISSUE HOMOGENATES

Organ		^{14}C-activity ($^{14}CO_2$)*					
		From Sevin I†			From Sevin II†		
		Counts/min	Equivalents of μg insecticide	% Metabolized Sevin	Counts/min	Equivalents of μg insecticide	% Metabolized Sevin
Blood (dil. once)		1400	8·2	13·7	200	2·0	2·8
Brain	(30%)	400	2·4	4·0	insign.	—	—
Liver		1120	6·6	11·0	120	1·2	1·7
Kidney		1020	6·0	10·0	120	1·2	1·7
Spleen		1150	6·8	11·3	80	0·8	1·1
Intestine		680	4·0	6·7	40	0·4	0·6

* The reaction mixture had the following composition: 1 ml phosphate buffer—0·1 M, pH 7·0; 1 ml tissue homogenate in isotonic KCl; 6 ml radioactive Sevin. Data are mean of four determinations.

† Absolute quantity of Sevin I/flask = 60 μg (10,200 counts/min);
Sevin II/flask = 72 μg (7200 counts/min).

TABLE 4. INHIBITION OF LIVER MONOAMINE OXIDASE BY SEVIN

System	Percentage* remaining activity
Homogenate + tyramine†	100
Homogenate + Sevin + tyramine (substrate added 15 sec prior to addition of Sevin)	96
Homogenate + Sevin + tyramine (substrate added 30 min after the addition of Sevin)	65
Homogenate + Sevin + tyramine (substrate added 60 min after the addition of Sevin)	48

* Results are mean of four determinations.
† Incubation period with tyramine—30 min.

for the elimination of Sevin metabolites; since the faeces contain only negligible radioactivity (less than 0·3 per cent of the administered dose). After 48 hr, 75–80 per cent of the injected dose could be recovered in the expired air and in the urine. The respiratory channel contributed 43·5 per cent of the recovered ^{14}C-activity; the rest being eliminated in the urine (cf. Table 1).

The production of $^{14}CO_2$ from Sevin I may be ascribed to the action of serum esterases which hydrolyse the ester bond of the insecticide. The liberated carbamic acid probably undergoes spontaneous decarboxylation.

The rate of elimination of $^{14}CO_2$ from Sevin II (Fig. 1) does not represent the true demethylation rate, because the eliminated $^{14}CO_2$ is not the only ^{14}C-metabolite produced as a result of demethylation. ^{14}C-formate is a second metabolite which accounts for about $\frac{1}{8}$ of the total ^{14}C-metabolites eliminated after 24 hr. In this connection, ^{14}C-formate is known to be excreted in urine, after the administration of ^{14}C-methanol[15] or ^{14}C-dipterex.[12] The products of demethylation ($^{14}CO_2$ and ^{14}C-formate) are eliminated in quantities almost equivalent to that of $^{14}CO_2$ produced by decarboxylation of N-methyl carbamic acid (Reaction Fig. 2). This suggests that N-methyl carbamic acid also constitutes a precursor for the demethylation products. This leads to the assumption that the intact molecule of Sevin is not demethylated to any significant extent. Direct evidence for the validity of this assumption is gained from the observation that urine from rats treated with Sevin I showed the presence of only one metabolite containing the carbon atom of the methyl group. In other words, it is the primary aliphatic amine released on decarboxylation, rather than Sevin itself, which becomes involved in the process of oxidative demethylation; probably via the MAO and catalase systems (equations 1–3). Into this picture, comes the inhibition of MAO in presence of Sevin. The progressive nature of inhibition suggests a very slow rate of dissociation of the enzyme-inhibitor compound.

The investigations carried out *in vitro* were aimed to provide information concerning the ability of the different organs to degrade Sevin. The hydrolysis of the ester bond proceeds at a much higher rate than the oxidative demethylation of the amine moiety (Table 3). This is because the first reaction is essentially a one-step reaction catalysed by a specific esterase. On the other hand, the production of $^{14}CO_2$ from $NH_2{}^{14}CH_3$ is

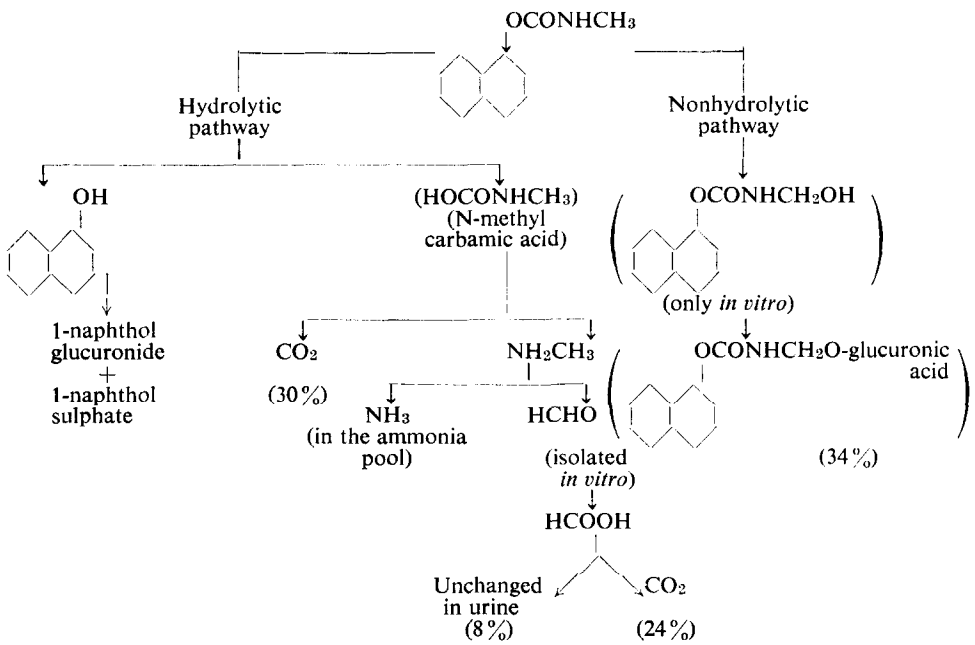
the result of a chain reaction, which presumably takes place in a fashion illustrated below:

$$NH_2{}^{14}CH_3 + O_2 + H_2O \xrightarrow{\text{amineoxidase}} H^{14}CHO + NH_3 + H_2O_2 \quad (1)$$

$$H^{14}CHO + O \xrightarrow{\text{aldehyde oxidase}} H^{14}COOH \quad (2)$$

$$H^{14}COOH + O \xrightarrow[\text{catalase } H_2O_2]{\text{catalase } H_2O_2} H_2O + {}^{14}CO_2. \quad (3)$$

Though the reaction is a multi-step reaction, it proceeds *in vitro* to the end product $^{14}CO_2$. This is explained on the basis that part of the H_2O_2 produced in the first step is utilized in the second and third steps for peroxidatic reactions. Without this source



Metabolite percentages—eliminated after 24 hr—are related to the original administered dose.

FIG. 2. Suggested scheme for the metabolic pathway of Sevin in the rat.

of peroxide, the reaction would never proceed to the ultimate product, since the oxidation of formate to carbon dioxide is catalysed by the catalase—H_2O_2 complex.[16] The low output of $^{14}CO_2$ may be explained on the basis that a part of the peroxide decomposes catalatically,[13] and probably also becomes involved in side reactions since tissue homogenates used in this investigation were undialysed. By arresting the reaction at the first step (by trapping $H^{14}CHO$), a higher metabolic rate has been observed. This also proves the formation of formaldehyde as intermediate in the degradation of Sevin. Though the nervous tissue contains a fairly high activity of amineoxidases, it is almost devoid of catalase.[17] This may explain the inability of brain homogenates to metabolize Sevin II to $^{14}CO_2$.

Colorimetric determination of phenols in urine showed the presence of a negligible amount of a free phenolic component. Paper chromatography of urine failed to detect free 1-naphthol. Since the enzymatic hydrolysis of the ester bond of the insecticide produces 1-naphthol, the latter must have been eliminated in a coupled form. Investigations by other authors, disclosed a general agreement that 1-naphthol is excreted almost completely in a conjugated form; possibly as ethereal sulphate and glucuronide.[18, 19] Since about 64 per cent of Sevin (1920 μg) was metabolized during the first 24 hr (Table 1), an equivalent amount of 1-naphthol (1376 μg) should have been eliminated in the urine. However, colorimetric determination accounted for only 960 μg. The incomplete recovery of 1-naphthol may be due to incomplete elimination of the naphthol and/or incomplete hydrolysis of the main metabolite in urine. Into this picture, comes the observation of Carpenter *et al.*[18] that less than $\frac{1}{3}$ of the naphthol content of Sevin was eliminated in the urine after 48 hr.

The nonhydrolytic pathway for the metabolism of Sevin gave a major metabolite (56·5 per cent of the total metabolite output) in the urine. Evidence for the presence of the skeleton C—O—C(O)—N—C has been presented. Degradation studies did not reveal the presence of phenolic products, other than 1-naphthol; indicating that the aromatic ring of the metabolite is not modified. It seems therefore justifiable to assume that the insecticide molecule has been modified through its methol group; by an oxidation process. In this case, it is believed that the methyl group is changed to a primary alcoholic group (—CH_2OH); and it is rather unlikely that oxidation proceeds beyond the alcohol stage. This assumption receives support from the work done by Dorough *et al.*[3] These authors reported that rat liver microsomes—fortified with reduced nicotinamide adenine dinucleotide phosphate (NADPH) or its oxidized form—degrade the methyl group of Sevin oxidatively. A major metabolite was isolated on a florisil column, and gave 1-naphthol on hydrolysis. The same authors claimed also that this metabolite decomposed under certain conditions to give 1-naphthyl carbamate. It is, therefore, believed that this metabolite and the ^{14}C-compound obtained *in vitro* (Table 2) are identical. It also constitutes an immediate precursor for the substance eliminated in the urine (Reaction Fig. 2). The possible formation of 1-naphthyl-N-methyl-N-hydroxy carbamate has been excluded.[3]

The strong hydrophilic nature of the main metabolite in urine suggests that the modified group is conjugated with a detoxifying agent. The reduction tests carried out on the paper chromatograms indicate that the coupling substance possesses a reducing group which is involved in conjugation. It is not unlikely that glucuronic acid is such a substance; which provides the metabolite with enough acidity to be excreted via the kidneys. In this case it is believed that the acid is linked with the alcohol through the hydroxyl group on carbon atom number 1 of the cyclic structure (glycoside type linkage). As a primary defence mechanism, glucuronic acid is known to couple with substances possessing an alcoholic group. In the light of this discussion, a formula has been suggested for the main metabolite excreted in urine (Reaction scheme 1).

Degradation studies give additional evidence concerning the nature of the conjugated metabolite. Whereas radioformate persists in alkaline medium, the conjugated metabolite suffers complete hydrolysis, and loses its ^{14}C-activity, as $^{14}CO_2$ from Sevin I, and possibly as $NH_2{}^{14}CH_2OH$ from Sevin II. In acid medium, the amine is trapped as a salt. The possibility that the main metabolite constitutes more than one substance of closely related structures and similar R_f values cannot be overruled.

It is believed that the liver and blood are the main participants in the detoxification of the insecticide. After 24 hr, the distribution pattern of radioactivity (expressed as μg insecticide) was: liver 160 μg; blood 98 μg; kidney 40 μg; spleen 16 μg; brain 8 μg giving total of about 11 per cent of the administered radioinsecticide

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