Nature of Certain Carbamate Metabolites of the Insecticide Sevin

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The chemical nature of Sevin metabolites formed by rat liver microsomes fortified with reduced nicotinamide–adenine dinucleotide phosphate and by cockroaches and houseflies was examined. Metabolites tentatively identified were 1-naphthyl *N*-hydroxymethylcarbamate, 4-hydroxy-1-naphthyl *N*-methylcarbamate, and 5-hydroxy-1-naphthyl *N*-methylcarbamate. At least two unidentified metabolites had the C—O—C(O)—N—C structure intact. Hydrolysis yielded 1-naphthol and at least two unidentified metabolites lacking the carbamyl group. These eight metabolites, five of which were carbamates, were formed by the liver microsomes and insects. Certain of these metabolites appeared in the milk of a goat treated orally with Sevin-carbonyl-C^{14}. Most of these metabolites were absent in plants injected with Sevin-C^{14} where carbonyl-C^{14}, methyl-C^{14}, or naphthyl-C^{14} samples of Sevin yielded water-soluble persisting metabolite(s). Similar, but more limited studies, are considered with Bayer 39007 (o-isopropoxyphenyl *N*-methylcarbamate). Bioassays on metabolites indicated reduced biological activity compared with the original insecticides.

The extensive use of Sevin (trademark, Union Carbide Chemicals Co.), 1-naphthyl *N*-methylcarbamate, has stimulated a variety of studies on the toxicology and residual persistence of this insecticide. The metabolic pathway of Sevin in organisms which it might contact in normal use has not been critically evaluated. Except for a single study concerning the metabolism of 1-naphthyl-1-C^{14} *N*-methylcarbamate in insects (*13*), only colorimetric and antiesterase types of analytical approaches have been employed.

A preliminary note on the present investigation reported that hydroxylation rather than hydrolysis may be the major detoxication mechanism for Sevin, since several carbamate metabolites are formed (*12*). The nature of these carbamate metabolites is considered in the present communication.

Experimental

Synthesis of Radiolabeled Carbamates. Sevin-carbonyl-C^{14}, Sevin-methyl-C^{14}, and *o*-isopropoxyphenyl *N*-methylcarbamate-carbonyl-C^{14} (Bayer 39007-carbonyl-C^{14}) were prepared according to a described procedure (*18*). Sevin-naphthyl-C^{14} was synthesized by reacting 1-naphthol-1-C^{14} with methyl isocyanate in the absence of catalyst or solvent. All products were purified by chromatography on Florisil columns (*18*), and their radiochemical purity was ascertained by thin layer chromatography as described later. The specific activity of all radiolabeled materials was 1 mc. per mmole or approximately 2500 c.p.m. per μg., as counted with the Model 314EX Packard Tri-Carb liquid scintillation spectrometer. In preliminary studies involving counting of aliquots from column eluates, the Nuclear-Chicago Model 183B scaler with a gas flow counter, utilizing a Micromil window, was employed.

Metabolism of Sevin by Rat Liver Microsomes. In a preliminary study (*12*), rat liver homogenates would not decompose Sevin to any appreciable extent unless the homogenates were fortified with nicotinamide-adenine dinucleotide phosphate (NADP) or its reduced form ($NADPH_2$). Fractionation studies showed that the effective system was the liver microsomes and $NADPH_2$.

For preparation of a standard microsome suspension, livers from freshly killed albino rats were homogenized in 0.05*M* sodium phosphate buffer (pH 7.3) to yield a 20% (w./v.) homogenate. The microsome fraction was considered to be that portion of the homogenate which was not precipitated by centrifugation at 15,000 G for 30 minutes, but was spun down at 105,000 G for 60 minutes. The microsome pellet was then homogenized in a phosphate buffer of sufficient volume to achieve reconstitution to the original 20% homogenate equivalent. Therefore, 1 ml. of the microsome suspension contained the microsomal fraction from 200 mg. of rat liver.

A typical incubation mixture contained 100 μg. of radiolabeled Sevin, 1 ml. of the microsome suspension, and 1 ml. of the phosphate buffer containing 2.0 μmoles of $NADPH_2$. The Sevin, in organic solvent, was transferred to a 25-ml. Erlenmeyer flask, and the solvent evaporated with care to deposit the Sevin uniformly over the bottom of the flask. After subsequent addition of the enzyme and cofactor solutions, the preparation was incubated in air with shaking at 37° C. for 2 hours.

Chromatography and Detection of Metabolites. Two column chromatographic systems were utilized. For chromatography on Florisil, a 2- × 30-cm. column was packed with 25 grams of Florisil (60/100 mesh, Floridin Co., Tallahassee, Fla.) from a slurry in hexane. The chromatogram was developed with the following solvent sequence—400 ml. of 1:1 hexane-ether, 300 ml. of 1:3 hexane-ether, 400 ml. of ether, and 200 ml. of methanol. Fractions, 20 ml., were collected at approximately 5 ml. per minute. A Celite column was also utilized where the stationary phase consisted of 20 ml. of acetonitrile saturated with hexane, mixed with 20 grams of Celite, and the mobile phase was hexane saturated with acetonitrile to which was then added 3% chloroform. The Celite

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column was used only for metabolites of Bayer 39007.

Thin layer chromatography (TLC) on Silica Gel G (Brinkmann Instruments, Inc., Great Neck, N. Y.) proved to be the most effective method of resolving Sevin and its metabolites. The plates were prepared 0.3 mm. thick for analytical studies and 1.0 mm. thick for preparative work. When the chromatograms were developed in only a single direction, a 4:1 ether-hexane mixture was usually used. For two-dimensional chromatography, the 20- × 20-cm. plates were first developed with 4:1 ether-hexane, and then after drying were developed in the other direction with 4:1 methylene chloride–acetonitrile unless otherwise stated. Radioactive materials on the plates were detected by radioautography, and the radioactive regions of the gel were scraped from the plate into scintillation vials for direct radioactive measurement. Some metabolites were unstable on the silica gel plates as discussed later.

A variety of chromogenic reagents were employed for detecting Sevin derivatives on the TLC. In addition to reagents previously reported (*18*), a solution of 1% ninhydrin in pyridine was used with a 30-minute color development period at 100° C. to detect as red spots the amines released on the decomposition of the carbamates.

Treatment of Insects, a Goat, and Plants with Radiolabeled Carbamates. In preliminary studies, adult female houseflies (*Musca domestica* L.) and adult female American cockroaches [*Periplaneta americana* (L.)] were treated topically at about 20 μg. per insect with acetone solutions of the three labeled samples of Sevin and the sample of Bayer 39007-carbonyl-C^{14}. Acetone extracts of the insects were prepared 4 hours after treatment. After the solvent was dried with sodium sulfate and the acetone evaporated off, the residues were chromatographed on Florisil. In a more detailed study, 35 cockroaches were injected through the ventral abdominal wall with 5 μg. of Sevin-carbonyl-C^{14} in 1 μl. of methyl cellosolve per roach. The roaches were confined in a chamber designed to trap the $C^{14}O_2$ expired during the experiment. Aliquots from the trap containing 30 ml. of a mixture of 2:1 methyl cellosolve–monoethanolamine were removed for direct liquid scintillation counting of expired $C^{14}O_2$ (*16*). In a final study with cockroaches, the same treatment procedure was utilized with 10 roaches receiving Sevin-carbonyl-C^{14}; another 10, the Sevin-methyl-C^{14}; and a final group of 10, Sevin-naphthyl-C^{14}. These roaches, which were injected with 5 μg. per roach of Sevin-carbonyl-C^{14} and Sevin-methyl-C^{14}, and 2 μg. per roach of Sevin-naphthyl-C^{14}, were also held in the chamber for trapping of expired $C^{14}O_2$. This dose of Sevin resulted in knockdown of the roaches but no mortality within a 24-hour period.

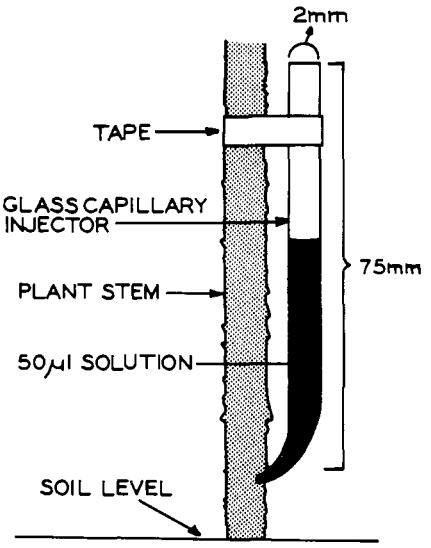


Figure 1. Capillary tube used to inject solution into plant stem for metabolism studies

A 55-kg. Saanen goat was catheterized and treated orally with Sevin-carbonyl-C^{14} at a rate of 1.34 mg. per kg. The labeled compound was distributed equally among four gelatin capsules containing a small amount of crushed corn to absorb the 2 ml. of chloroform involved in the transfer. A balling gun was used to administer the capsules. This dose of Sevin had no apparent adverse effects on the goat. Milk and urine were collected at frequent intervals up to 96 hours after treatment so that aliquots representative of the total amount could be analyzed.

Garden snapbeans (Contender variety) and cotton (Lankart 55 variety) seedlings were treated with Sevin-C^{14} and Bayer 39007-C^{14} by injection of the labeled material through the stem. An acetone-water solution (10:90) of the radiolabeled carbamate in a microsyringe was slowly introduced into a capillary tube with care to prevent the trapping of air bubbles in the tube. The fine tip of this tube was then inserted into the stem (Figure 1). In most cases, the 50-μl. volume was taken up by the plant in 30 to 45 minutes, after which time the glass tube was removed. A dose of 40 μg. or about 100,000 c.p.m. of labeled carbamate was introduced into each plant in this manner.

Extraction of the Carbamates and Their Metabolites. With the microsome preparations, the incubation mixture was either evaporated directly onto a small amount of Florisil which was added to the top of the Florisil column for chromatographic development, or extracted four times with 5-ml. portions of ether. The ether extract was dried with anhydrous sodium sulfate and the ether then evaporated either onto Florisil for column chromatography or to approximately 100-μl. total volume for application by microsyringe to the TLC.

Two procedures were used for extraction of the insects. For extraction with acetone, either five cockroaches or 200 flies were homogenized with a Lourdes Multimixer in 50 ml. of acetone and filtered, and the residue again was extracted with an additional 50 ml. of acetone. The acetone extracts were combined and dried with sodium sulfate, and the volume was reduced to approximately 5 ml. A small amount of Florisil was then added to the extract, the solvent evaporated to dryness on the Florisil, and the powder added to the top of the Florisil column for development of the chromatogram. An alternate procedure which allowed a higher percentage recovery of administered radioactivity involved homogenizing two cockroaches in 50 ml. of water with the Lourdes Multimixer. After thorough homogenization, the brei was transferred to a 250-ml. separatory funnel. One-hundred milliliters of acetonitrile was then added to the separatory funnel and the mixture shaken. Insoluble materials were removed by filtration through a coarse filter paper into a second 250-ml. separatory funnel. To the filtrate was added 100 ml. of chloroform and the solvents were thoroughly mixed. After separation of the phases, the upper aqueous phase was rewashed with 50 ml. of chloroform which was combined with the first organic extract. The combined acetonitrile-chloroform extract was dried with sodium sulfate, the solvents were removed under reduced pressure, and the residue was dissolved by washing twice with 25-ml. portions of acetone. The acetone was evaporated by a gentle air stream to a volume of about 10 ml. and cooled to −20° C., at which temperature a precipitate formed (containing no radioactivity) and was readily removed by filtration through a plug of glass wool. The clear acetone was then evaporated to a volume suitable for spotting on the TLC. With this acetonitrile-chloroform extraction procedure, over 95% of the administered compound was always recovered, either in the water solubles (not extracted from water by organic solvents) or in the radioactive fractions recovered from TLC or as expired $C^{14}O_2$.

The goat milk was extracted by a described procedure (*22*) within 1 hour following milking, and analyzed on a Florisil column. One-hundred milliliters of fresh milk was placed in a 500-ml. separatory funnel and 150 ml. of acetonitrile added. The mixture was thoroughly shaken until a solid precipitate formed. In some cases, the mixture had to be warmed slightly to obtain complete coagulation of the milk proteins. The acetonitrile-water was separated from the precipitate by de-

canting into a second 500-ml. separatory funnel. The milk solids were washed with 100 ml. of chloroform which was added to the acetonitrile-water mixture. After shaking, the acetonitrile-chloroform layer was removed and the aqueous layer re-extracted with 70 ml. of 1:1 acetonitrile-chloroform. The combined organosoluble or solvent-extractable phases were dried with sodium sulfate, the total p.p.m. Sevin-C^{14}-equivalents determined, and the components of this extract separated by chromatography on Florisil. The p.p.m. Sevin equivalents in the fresh whole milk were determined by direct counting of 0.2-ml. aliquots. Aliquots of the water (0.2 ml.) and milk solids (20 mg., suspension) were used for counting to ascertain the p.p.m. Sevin equivalents in these fractions. Considerable variation occurred in the number of counts between these two fractions, in part due to differences in the ease of coagulation of the milk proteins. It is not known to what degree cross-contamination of these fractions occurred, or even whether chemically distinct materials were present in the two fractions. To evaluate the extraction efficiency for the Sevin metabolites by this procedure, the same metabolites formed by rat liver microsomes were assumed to be present in the milk from the treated goat. Accordingly, metabolites of Sevin formed by the microsomes were separated on the Florisil column and then individually used to fortify control milk samples which were then subsequently carried through the procedure. In each case, recovery of the individual metabolites exceeded 90%.

One extraction procedure for the treated plants involved homogenization of individual plants in 50 ml. of acetone, filtration and drying of the acetone extract with sodium sulfate, and evaporation of the acetone onto a small amount of Florisil for chromatography. In a second procedure, the plant was homogenized in acetone and the plant residue re-extracted with 100 ml. of chloroform. The two extracts were combined and thoroughly mixed, and the organic solvent was separated from the aqueous layer. The acetone-chloroform mixture was then washed twice with 20-ml. portions of water, and these washes were added to the original water fraction. This second procedure allowed the separation of the radiolabeled materials into organo- and water-soluble products, depending on their partitioning characteristics.

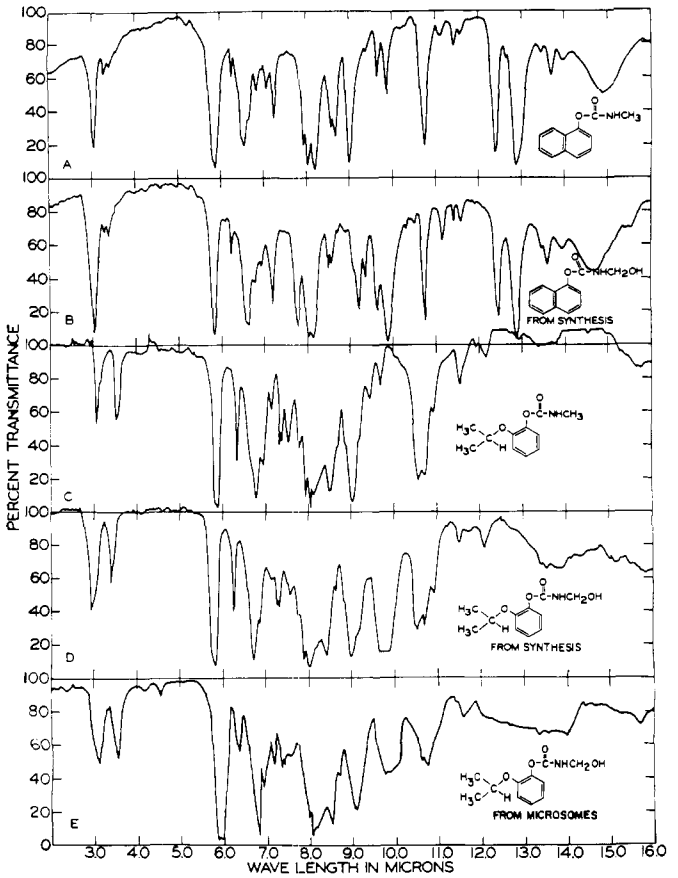
Formation of Radiolabeled Derivatives from Degradation Products of Sevin-C^{14} Metabolites. The conditions for hydrolysis of the organosoluble carbamate metabolites of Sevin were ascertained with Sevin-carbonyl-C^{14}. Complete hydrolysis resulted on evaporating the individual metabolite fractions to dryness, adding 5 ml. of 1*N* sodium hydroxide, holding at 100° C. for 30 minutes, and cooling and adding dilute hydrochloric acid to pH 2. Extraction of this aqueous solution twice with 10-ml. portions of chloroform resulted in recovery of the ring fragments in the organic phase and the methyl fragments in the water phase.

To determine the nature of the ring fragment, 20 mg. of technical 1-naphthol was added to the chloroform, and the mixture was evaporated to dryness with the aid of a gentle air stream. Methyl isocyanate was added in excess and allowed to stand overnight at room temperature in a stoppered flask. The excess methyl isocyanate was then evaporated and the contents of the flask transferred to a Florisil column. When the ring-labeled metabolite yielded 1-naphthol-C^{14} upon hydrolysis, Sevin-naphthyl-C^{14} was recovered after reaction with methyl isocyanate. When the ring had been modified during metabolism, the radiolabeled parent compound was not recovered by such a procedure.

Metabolites from Sevin-methyl-C^{14} were used for determination of radiolabeled methylamine or formaldehyde. The methyl-C^{14} metabolite was placed in a 50-ml. pear-shaped flask and the solvent evaporated. For determination of methylamine-C^{14}, 10 ml. of 2*N* sodium hydroxide was then added and the flask fitted with a water-cooled distillation condenser leading to a receiver cooled in an ice bath. The alkaline solution was heated until almost all of the water had distilled over. To a 1-ml. aliquot of the distillate containing the labeled methylamine was added 20 µl. of 40% aqueous nonlabeled methylamine followed by 42 µl. of phenylisothiocyanate. Crystals of methylphenylthiourea were formed after violent shaking of the mixture. The radioactive content of the crystals was determined directly by liquid scintillation counting, and the thiourea was also subjected to TLC. Methylphenylthiourea was detected by spraying with a 1% aqueous solution of ferric chloride followed by a 1% aqueous solution of potassium ferricyanide to yield a blue spot. On development of the TLC with a 2:1 ether-hexane mixture, the methylphenylthiourea yielded an R_f of about 0.5. The coincidence of the radioactive material with methylphenylthiourea was determined by radioautography. For determination of radiolabeled formaldehyde, 10 ml. of cold 2% sulfuric acid was added to the flask containing the methyl-C^{14} metabolite. The flask was fitted with a water-cooled distillation condenser leading to an ice-cold receiving flask, and distillation was allowed to proceed until the water was almost entirely distilled over. If the aqueous distillate contained radioactive material, which was assumed to be formaldehyde, it was fortified with 10 µl. of a nonradioactive 40% aqueous solution of formaldehyde. Next, 25 ml. of a hot 0.25% aqueous solution of 4-hydroxycoumarin was added. White crystals of dicoumarol began to form immediately, and the reaction was carried to completion by gentle boiling for 1 hour (*20*). The crystals were removed by filtration from the hot solution. The dicoumarol was counted directly for radioactive content, or spotted on TLC and developed with 95% ethanol. It could be detected (R_f 0.72) by spraying with a saturated hexane solution of iodine or by radioautography.

Attempted Synthesis of Possible Metabolites Involving Modifications on the Nitrogen or Methyl Groups. The chloroformates of 1-naphthol and *o*-isopropoxyphenol were synthesized according to a described procedure for this type of compound (*24*). These chloroformates were used in the preparation of various substituted carbamates by adding the chloroformate to an aqueous solution of the amine, shaking vigorously for 30 minutes, and recovering the products by extraction with chloroform. Each product was purified by chromatography on Florisil and recrystallized where possible. The infrared spectra of the products were consistent with the proposed structures. Unsubstituted carbamates were prepared by adding the chloroformates to aqueous ammonium hydroxide. The *N*-methyl, *N*-hydroxycarbamates and the *N*-methoxycarbamates were made by adding 5 mmoles of sodium hydroxide to 5 mmoles of the amine hydrochloride in 4 ml. of water. The chloroformate was subsequently added at 2.5 mmoles with the reaction conditions and method of product recovery as previously indicated.

A variety of procedures were attempted to prepare the *N*-hydroxymethylcarbamates. Only one method (suggested by J. R. Kilsheimer of Union Carbide Chemicals Co., South Charleston, W. Va.) was encouraging, but this procedure gave poor yields of the presumed product. To 1-naphthyl carbamate (0.25 mole) in 200 ml. of glacial acetic acid was added 0.25 mole of paraformaldehyde at room temperature. The reaction mixture was slowly raised to 90° C. and this temperature held for 1 hour. After stripping the acetic acid at room temperature, the residue was dissolved in ether and washed thoroughly with water. The product obtained on stripping the ether (42 grams) was found by TLC to be a mixture of seven components. One minor constituent, suspected from its chromatographic characteristics of being 1-naphthyl *N*-hydroxymethylcarbamate, was separated by preparative scale TLC. One-hundred milligrams of the technical 1-naphthyl carbamate–paraformaldehyde reaction product was placed as a band



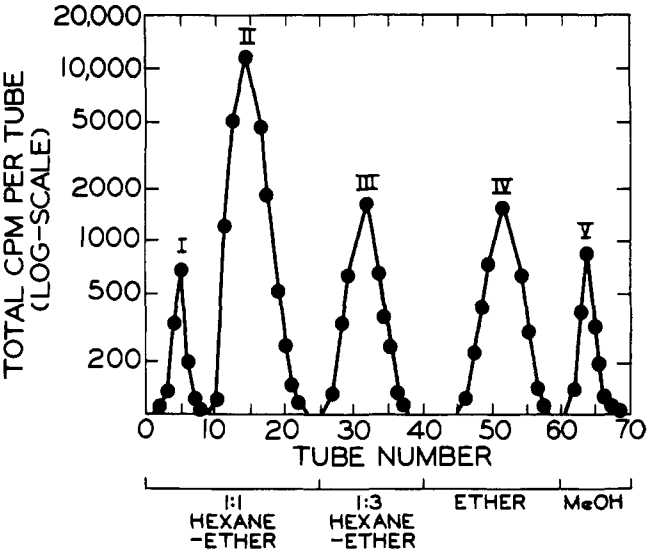


Figure 3. Sevin-naphthyl-C^{14} metabolites formed by liver microsomes as separated on Florisil column using unextracted reaction mixture

Roman numerals indicate designations for peaks as used in text; eluate fractions were counted on gas flow counter; recovery of total counts utilized ranged from 90 to 95%

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Figure 2. Infrared spectra for Sevin and Bayer 39007 (*A* and *C*), their *N*-hydroxymethyl analogs from synthesis (*B* and *D*), and one metabolite of Bayer 39007 from microsomes (*E*)

Spectra *A* and *B* from KBr pellets; spectra *C*, *D*, and *E* from 10% solutions in chloroform; Baird Model 4-55 infrared spectrometer, sodium chloride optics

at the origin of each of several 20- × 20-cm. thin layer plates which had been prepared with silica gel of 1.0-mm. thickness. These chromatograms were developed with 4:1 methylene chloride–acetonitrile. Two fluorescent regions were evident on examining the developed chromatograms under short-wave ultraviolet light. The major band appeared close to the front, and the minor band between R_f 0.15 and 0.25 was recovered from the chromatograms for closer examination. The fluorescent region was scraped from the chromatograms, the silica gel extracted with ether, the ether evaporated, and the carbamate crystallized by the addition of hexane to the ether. The resulting product constituted about 2% of the original technical carbamate and consisted of a single component based on two-dimensional TLC. This material, melting point 137–139° C., had an infrared spectrum consistent with that of the *N*-hydroxymethyl product desired (for comparison with spectrum of Sevin, see Figure 2). On degradation, it yielded formaldehyde and 1-naphthol. Analyses (S. M. Nagy, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass.) gave 66.07% carbon, 5.29% hydrogen, 6.47% nitrogen, and molecular weight 217, compared with theory for $C_{12}H_{11}NO_3$ of 66.35% carbon, 5.11% hydrogen, 6.45% nitrogen, and molecular weight 217.

o-Isopropoxyphenyl carbamate was also reacted with paraformaldehyde under similar conditions but on a much smaller scale. Purification on the Florisil column yielded a component eluting with 3:1 ether-hexane, which decomposed to yield formaldehyde under strong acid conditions. The infrared spectrum of this crystalline product was consistent with *o*-isopropoxyphenyl *N*-hydroxymethylcarbamate (for comparison with spectrum of Bayer 39007, see Figure 2).

Attempted Synthesis of *N*-Methylcarbamates with Modifications on the Naphthalene Ring. Preliminary attempts to modify the ring or *N*-methyl group by oxidation of Sevin or Bayer 39007 with potassium permanganate, hydrogen peroxide, or peracetic acid proved unsuccessful. A number of substituted 1-naphthyl *N*-methylcarbamates were therefore synthesized for comparison with radiolabeled metabolites of Sevin. The following dihydroxynaphthalenes were reacted under mild conditions with methyl isocyanate: 1,2; 1,3; 1,4; 1,5; and 1,7. The purified product from the 1,2-dihydroxynaphthalene reaction was provided by H. A. Stansbury, Union Carbide Chemicals Co., South Charleston, W. Va. The 1,4-dihydroxynaphthalene was prepared by reduction of 1,4-naphthoquinone (*14*). About 100 mg. of the various dihydroxynaphthalenes and about 0.2 ml. of methyl isocyanate were sealed in an ampule and allowed to react at room temperature for up to 4 hours. After completion of the reaction, the methyl isocyanate was evaporated with an air stream. The residue was dissolved in acetone and the composition evaluated by TLC. The monocarbamates in the reaction mixtures were considered to be those products which, when separated by TLC, responded to chromogenic reagents for phenols prior to hydrolysis and in addition gave a positive test with ninhydrin-pyridine for methylamine. The dicarbamates failed to respond to the phenolic test prior to hydrolysis but gave the ninhydrin-amine test, and following alkaline hydrolysis, the phenolic tests were positive. The unreacted dihydroxynaphthalenes failed to give a red spot with the ninhydrin test. The monocarbamate products from the 1,2- and 1,5-dihydroxynaphthalenes were separated and examined by infrared spectroscopy and found to contain both hydroxyl and carbonyl functions. To obtain the monocarbamate from 1,4-dihydroxynaphthalene, it was necessary to reduce the reaction time to 30 minutes or less. Two monocarbamates would be expected with the 1,2-, 1,3-, and 1,7-dihydroxynaphthalenes, whereas a single

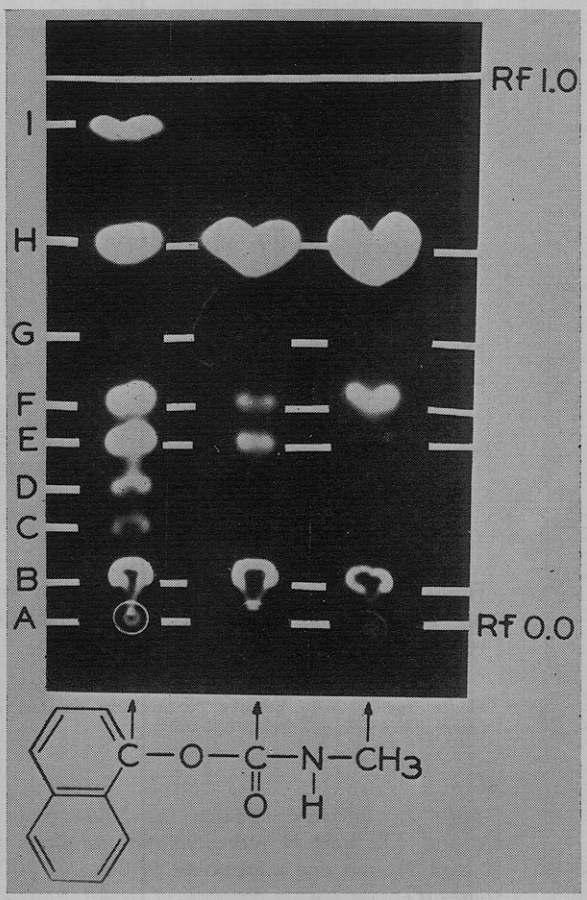

Figure 4. Sevin-C^{14} metabolites formed by liver microsomes as separated by thin layer chromatography (4 : 1 ether-hexane) using ether extracts of reaction mixtures

Site of carbon^{14} label for substrate in each reaction mixture as indicated by structural formula; metabolites as detected by radioautography; A–I indicate designations for metabolites as used in text

monocarbamate should form with the 1,4- and 1,5-dihydroxynaphthalenes. Radioautography was used to ascertain whether any of the monocarbamates from these reaction mixtures cochromatographed with radiolabeled metabolites of Sevin.

A sample of 1,2-dihydro-1,2-dihydroxynaphthalene (kindly provided by L. C. Terriere of Oregon State University, Corvallis, Ore.) was also reacted with methyl isocyanate in the same manner. The products as separated by TLC were detected utilizing the ninhydrin-pyridine reagent. Two ninhydrin-positive components were formed during this reaction.

Other Methods. Chromotropic acid was used for determination of formaldehyde released on acid decomposition (*10*) of certain carbamates. The conditions for gas chromatography of *o*-isopropoxyphenol were as previously reported (*18*). Cholinesterase assays were made by determining the rate of carbon dioxide evolution resulting from acid liberation on acetylcholine hydrolysis in a bicarbonate buffer (0.0357*M* sodium bicarbonate and 0.164*M* sodium chloride) with an atmosphere of 5% carbon dioxide and 95% nitrogen at 38° C. in 10-ml. Warburg flasks. For determination of anticholinesterase activity, the inhibitors in acetone were evaporated on the bottom of the Warburg flask, and 1.6 ml. of a homogenate containing three fly heads in buffer were then added. The enzyme and inhibitor were incubated together for 30 minutes at 38° C. prior to addition of the substrate. Acetylcholine in 0.4-ml. volume was tipped from the side arm at zero time to yield a final concentration of $1 \times 10^{-2}M$. Only initial reaction velocities (first 10 minutes) were considered for interpretation of results. For bioassays, 4-day-old adult female house flies (C.S.M.A., 1948 strain) were treated on the ventrum of the abdomen with 1 μl. of acetone containing the test substance. In all toxicity studies reported, the flies were treated first with 10 μg. of piperonyl butoxide per insect and immediately afterwards with the carbamate. Seven-gram female white mice from the Rolfsmeyer Farms (Madison, Wisconsin) were treated intraperitoneally with 0.1 ml. of propylene glycol containing the carbamate.

Results

Metabolism of Sevin by Liver Microsomes. Chromatography of the microsome incubation mixtures on a Florisil column yielded three or four major peaks attributable to metabolites of Sevin-C^{14}, the number of metabolites depending on the position of the C^{14} label. For the most complete recovery of the radioactivity from the microsome incubation mixtures, the entire 2-ml. volume was evaporated to dryness on a small amount of Florisil and then transferred to the Florisil column for development. Results by this technique with Sevin-naphthyl-C^{14} are indicated in Figure 3. When this experiment was repeated with Sevin-carbonyl-C^{14} or Sevin-methyl-C^{14}, the results were essentially the same except that peak I was absent.

Cochromatographic studies on Florisil established that peak I was 1-naphthol and peak II was Sevin. It appeared likely that the metabolites appearing in peaks III, IV, and V had the C—O—C(O)—N—C skeleton intact as these metabolites appeared in about the same proportion with each of the different labeled substrates. Each of the metabolite peaks from the Florisil was found to maintain its chromatographic integrity after isolation and return to the Florisil column. After alkaline hydrolysis of the separated metabolite peaks from Sevin-naphthyl-C^{14}, acidification, recovery of the ring-labeled hydrolysis product(s) in chloroform, and reaction with methyl isocyanate, the hydrolysis product of peak II yielded Sevin as expected. The hydrolyzate of peak III, after reaction with methyl isocyanate, gave Sevin as the major radioactive product, plus a small amount of labeled material which had the original peak III chromatographic position. The major portion of peak III thus yielded 1-naphthol on hydrolysis, but another material(s) was present which had a modified ring structure. The hydrolyzed product of peak IV reacted with methyl isocyanate to give a product which chromatographed on the Florisil column in the exact position of the original metabolite. This was interpreted as meaning that peak IV was a metabolite of Sevin with a ring modification, but no modification of the methylcarbamyl group. The chromatographic position of peak V also remained the same after the above treatment and the metabolites thus probably contained a ring modification.

Thin layer chromatography yielded evidence that the metabolism picture was more complex than that indicated by chromatography on Florisil columns. Ether extractables from the incubation

mixtures yielded radioautograms from the TLC as indicated in Figure 4. Sevin-naphthyl-C^{14} plus microsomes and $NADPH_2$ yielded seven metabolites other than the material remaining at the origin. Sevin per se appeared as spot H. The coincidence of metabolite positions with the different labeled substrates shows that metabolites B, E, F, and G had the C—O—C(O)—N—C skeleton intact and that relatively little hydrolysis took place in the microsome incubation mixture. The material at the origin, metabolite A, was present with each of the labeled samples of Sevin and probably represented a carry-over of polar products from the aqueous layer. Metabolites C, D, and I were present only with Sevin-naphthyl-C^{14} and were therefore hydrolysis products, although it is not clear whether they were formed by initial hydrolysis of Sevin and then further degradation of the naphthol, or by initial ring modifications on Sevin followed by hydrolysis. Metabolite I was identified as 1-naphthol by TLC cochromatography and by reaction with methyl isocyanate to yield Sevin. The origin of metabolite C remains unknown, but there were indications that metabolite D was a decomposition product of metabolite B.

Comparison of the metabolites from the Florisil column with the spots on the TLC showed that metabolite A would appear in peak V and metabolite B was the only constituent of peak IV. Metabolites C and D were in amounts too small to be detected by the column type of analysis. Metabolites E, F, and G appeared as a mixture within peak III. Sevin, designated as H, eluted as peak II, and naphthol, or metabolite I, appeared as the first peak on Florisil. Therefore, analysis by the Florisil column would give results for a single material in peak IV but a mixture of three materials in peak III. The Florisil column was accordingly used for cleanup in cases where the extract could not be applied directly to the TLC for resolution of the metabolites.

The extent of conversion of Sevin to various metabolites by rat liver microsomes is indicated in Table I. This experiment was designed to determine if each of the labeled samples of Sevin gave similar results under identical incubation conditions. Although there is some variation in the percentage of the individual metabolites recovered, the proportion of the recovered radioactivity appearing in each metabolite containing the carbamyl group was essentially the same. Because the per cent radioactive material in the water solubles did not vary greatly with different radiolabeled sites on the carbamate, this fraction, at least in part, apparently had the C—O—C(O)—N—C skeleton intact. It was difficult to obtain reproducible results by this incubation procedure, as indicated by the fact that the amount of Sevin after incubation varied in different studies from 20 to 80%. A portion of this variation may have been due to differences in enzymatic activity of the microsome preparations. A further difficulty resulted from the poor solubility of Sevin in water which necessitated depositing the material on the incubation flask by evaporation of an organic solvent which may have yielded variations in the deposit and therefore in the rapidity with which the substrate and enzymes were able to combine. When the same batch of microsomes was used for separate incubation mixtures within a single experiment, the results were quite reproducible (Table I).

Table I. Recovery of Various Sevin Metabolites as Separated by Thin Layer Chromatography from Incubation Mixtures of Sevin-Carbonyl-C^{14}, Sevin-Methyl-C^{14}, or Sevin-Naphthyl-C^{14} with Rat Liver Microsomes

	% of Initial C^{14} Present as:		
Metabolites	*Carbonyl-* C^{14}	*Methyl-* C^{14}	*Naphthyl-* C^{14}
Water-soluble	6.7	7.3	6.8
Ether-soluble			
A	0.5	0.6	0.6
B	2.4	3.3	2.1
C	...	...	0.3
D	...	...	0.4
E	3.7	4.2	3.3
F	1.3	2.0	1.9
G	0.6	0.7	0.7
H (Sevin)	78.4	79.0	81.9
I	...	...	1.7
Total % recovery	93.6	97.1	99.7

Stability of Sevin Metabolites on Silica Gel Thin Layer Chromatograms. Difficulties were encountered in extraction of the Sevin metabolites from the silica gel. Acetonitrile proved to be the most satisfactory solvent for such an extraction although less than 50% recovery was obtained with most of the metabolites. Rechromatography on TLC of the material extracted from the silica gel showed that each of the metabolites had undergone some degree of decomposition and now appeared as two or more components except for Sevin per se, which maintained its original chromatographic position. Two-dimensional chromatography was utilized to establish further that decomposition was occurring on the silica gel. Metabolites from Sevin-naphthyl-C^{14} were developed in the first direction with 4:1 ether-hexane. The plate was removed and immediately upon drying was developed in the second direction with the same solvent system. Each of the metabolites remained as a single component as ascertained by radioautography. However, if the plate was allowed to stand overnight at room temperature before the second development, considerable decomposition occurred with all materials except Sevin. Each metabolite yielded products which remained at the origin, and metabolites E, F, and I also decomposed into other products which moved away from the origin. Metabolites C and G had completely decomposed to yield materials with different chromatographic positions. An identical experiment was repeated with metabolites from Sevin-carbonyl-C^{14} and Sevin-methyl-C^{14}. The results were the same as for the naphthyl-labeled material except with metabolite E formed from Sevin-methyl-C^{14}. A degradation product evident with the carbonyl- and naphthyl-labeled E metabolites was not detected with the same metabolite when labeled in the methyl group. This indicated that degradation on the silica gel resulted in loss of the *N*-methyl carbon, possibly to yield 1-naphthyl carbamate. Cochromatography of the radioactive degradation product with a known sample of 1-naphthyl carbamate confirmed this assumption. The difficulties in recovering the metabolites from silica gel by extraction resulted from decomposition rather than poor extraction efficiency. When the developed thin layer chromatograms were held at −20° C. overnight while being exposed for the radioautogram, the metabolites did not decompose and direct ether extraction of the silica gel gave almost quantitative recovery of the metabolites.

Chemical Nature of the Carbamate Metabolites of Sevin from Microsomes. Metabolites of Sevin-naphthyl-C^{14} were used to evaluate possible modifications in the ring. Metabolites B, E, F, G, and H (Sevin) as recovered from TLC were hydrolyzed in alkali. The ring fragments were then recovered from the hydrolyzates by acidification and extraction into chloroform, and subjected to TLC fractionation. The only materials yielding 1-naphthol on hydrolysis were Sevin and metabolite E. Evidently metabolites B, F, and G contained ring modifications of some type. The hydrolyzed material of metabolite B had the same chromatographic position as metabolite D, which, as already noted, was a derivative of 1-naphthol lacking the carbamyl group. Possibly metabolite B is the precursor of metabolite D.

The various metabolites from Sevin-methyl-C^{14} were examined for possible modification of the methyl group. The *N*-methylcarbamyl group would be expected to yield methylamine-C^{14} on hydrolysis, and the *N*-hydroxymethylcarbamyl group to yield formaldehyde-C^{14} on degradation. Metabolites B, E, F, and Sevin were thus examined. Sevin and metabolite B yielded methylamine upon hydrolysis, and formaldehyde was detected from metabolite E. The

Table II. Response of Sevin Metabolites from Thin Layer Chromatograms and Several Synthetic Analogs to Chromogenic Reagents[a]

Compound[b]	Gibbs'		$FeCl_3$–$K_3Fe(CN)_6$		Fluoborate	Alkoli-Fluoresence
	$-OH^-$	$+OH^-$	$-OH^-$	$+OH^-$	$+OH^-$	
1-Naphthol	blue	blue	blue	blue	yellow	+
Metabolite I	blue	blue	blue	blue	yellow	+
1-Naphthyl *N*-methylcarbamate	...	blue	...	blue	yellow	+
Metabolite H	...	blue	...	blue	yellow	+
5-Hydroxy-1-naphthyl *N*-methylcarbamate	blue	blue	blue	blue	yellow	+
Metabolite G	blue	blue	blue	blue	yellow	+
4-Hydroxy-1-naphthyl *N*-methylcarbamate	blue	blue	blue	blue	orange	+
Metabolite F	blue	blue	blue	blue	orange	+
1-Naphthyl *N*-hydroxymethylcarbamate	...	blue	...	blue	yellow	+
Metabolite E	...	blue	...	blue	yellow	+
Metabolite D	blue[c]	blue[c]				
Metabolite B	...	blue[c]	...	blue[c]	...	−
2-Hydroxy-1-naphthyl *N*-methylcarbamate	orange	blue	blue	blue	orange	+
3-Hydroxy-1-naphthyl *N*-methylcarbamate	blue	blue	blue	blue	orange	+
7-Hydroxy-1-naphthyl *N*-methylcarbamate	blue	blue	blue	blue	orange	+
1,2-Dihydro-1,2-dihydroxynaphthalene	...	...	...	...	orange	−
1,2-Dihydro-2-hydroxy-1-naphthyl *N*-methylcarbamate	...	...	...	...	orange	−

[a] Chromogenic reagents were applied to the thin layer chromatograms on which the metabolites or synthetic analogs were separated. The amount of compound in the spot after separation was about 0.3 to 3 μg. A negative response (−) indicates that the spot was not detected with about 10 μg. of compound. The composition of the indicated spray reagents has been reported (*18*).

[b] All the synthetic compounds and metabolites containing the carbamyl grouping gave a red color with pyridine-ninhydrin as indicated in text. All of the metabolites indicated except B and D gave a red color on spraying with alkali followed by diazotized sulfanilic acid reagent. All metabolites indicated gave a red color on spraying with alkali, 4-aminoantipyrine, and finally potassium ferricyanide.

[c] About 10 μg. of metabolite B were required for these responses. Insufficient material was available for testing metabolite D with other than Gibbs' reagent.

results with metabolite F were inconclusive, although indications were obtained for methylamine and only negative tests for formaldehyde.

Except for 1-naphthol, the only Sevin metabolite with an unmodified ring structure was E. Based on analogy with studies on *N*,*N*-dimethylcarbamates (*15*), this product was suspected to be 1-naphthyl *N*-hydroxymethylcarbamate, particularly since it decomposed in acid to yield formaldehyde or on the TLC to 1-naphthyl carbamate. An attempt to prepare 1-naphthyl *N*-hydroxymethylcarbamate yielded a technical material which on TLC was found to consist of a mixture of at least seven components. Two-dimensional TLC of the mixture combined with the C^{14}-labeled metabolites showed that one of the products was coincident with metabolite E on cochromatography. When this small component was isolated by preparative scale TLC, it was found to be 1-naphthyl N-hydroxymethylcarbamate based on a variety of characteristics described above in the section on attempted synthesis of metabolites. Further, the synthetic material cochromatographed both on Florisil and two dimensional TLC with metabolite E. This radiolabeled metabolite of Sevin failed to cochromatograph with 1-naphthyl *N*-methyl, *N*-hydroxycarbamate, or 1-naphthyl *N*-methoxycarbamate, and these two synthetic materials failed to yield formaldehyde on acid degradation. Metabolite E of Sevin thus appeared to be 1-naphthyl *N*-hydroxymethylcarbamate. Low yields in the rat liver microsome system did not permit isolation of sufficient metabolite E for direct spectral and analytical examination.

The available evidence on metabolites B, F, and G indicated that these materials consisted of a modified ring structure with the *N*-methylcarbamyl group unchanged. Studies on the metabolism of naphthalene showed that the ring can be modified to form 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol (*1*, *3–5*, *19*, *21*). The nature of the enzymatic system in which these metabolites were formed also suggested oxidation or hydroxylation of the ring. Reaction mixtures containing various hydroxy-1-naphthyl *N*-methylcarbamates were mixed with the ether-soluble fraction from a Sevin-naphthyl-C^{14} microsome preparation. These materials were spotted on TLC and developed two dimensionally with the first solvent a 4:1 ether-hexane mixture and with the second, a 4:1 methylene chloride–acetonitrile mixture. The chromatogram was sprayed with ninhydrin to locate the carbamates, and the radioactive metabolites were located by radioautography. This experiment revealed that a monocarbamate, presumed to be 4-hydroxy-1-naphthyl *N*-methylcarbamate, cochromatographed with metabolite F, and another, presumed to be 5-hydroxy-1-naphthyl *N*-methylcarbamate, cochromatographed with metabolite G. None of the other products from the reaction of any of the dihydroxynaphthalenes cochromatographed with any of the radiolabeled metabolites. The major product from the reaction of 1,2-dihydro-1,2-dihydroxynaphthalene and methyl isocyanate cochromatographed with metabolite B with ether-hexane as a solvent but was separated with methylene chloride–acetonitrile. This was the only product that had the general chromatographic position of metabolite B, indicating that this type of modification of the naphthol ring might be involved.

Further evidence that metabolite F was 4-hydroxy-1-naphthyl *N*-methylcarbamate and metabolite G was 5-hydroxy-1-naphthyl *N*-methylcarbamate resulted from studies where colored derivatives were obtained with the synthetic products when sprayed with Gibbs' reagent. These blue derivatives had different chromatographic positions on TLC than the unreacted carbamates. A mixture of the C^{14} metabolite and the synthetic hydroxy compound was spotted on TLC and then sprayed with Gibbs' reagent. When the chromatogram was developed with 4:1 ether-hexane, the R_f for the colored derivative from 4-hydroxy-1-naphthyl *N*-methylcarbamate (0.90) was greater than that of the original material (0.40), and the R_f of the 5-hydroxy Gibbs' derivative (0.44) was lower than that of the original 5-hydroxy-1-naphthyl *N*-methylcarbamate (0.50). Radioautography showed that the radioactivity cochromatographed with the blue derivatives from Gibbs' reagent and did not appear in the original regions for metabolites F and G. Gibbs' reagent thus converted metabolite F and 4-hydroxy-1-naphthyl *N*-methylcarbamate to the same derivative (R_f 0.90), and metabolite G and 5-hydroxy-1-naphthyl *N*-methylcarbamate to the same derivative (R_f 0.44).

Chromogenic reagents were also useful in ascertaining the nature of the Sevin metabolites formed by rat liver microsomes. The response of the Sevin metabolites and certain synthetic analogs as separated by TLC is indicated in Table II. Metabolites E, H, and I,

Table III. Per Cent of Various Sevin Metabolites from Cockroaches at Various Times after Injection of Sevin-Carbonyl-C^{14} Based on Extraction with Acetone and Chromatography on Florisil

Hours after Treatment	Per Cent of Administered Radioactivity Present as:			
	Sevin	Peak III[a]	Peak IV[b]	CO_2
0	89.0	0	0	0
1	42.5	7.7	1.3	0.3
2	31.1	10.2	2.4	1.0
4	20.2	11.2	2.5	3.3
8	14.0	10.3	4.4	8.9
12	10.0	7.9	3.9	13.2
24	6.0	4.1	1.9	19.3

[a] A mixture of metabolites E, F, and G.
[b] Metabolite B.

vhich appeared to be 1-naphthyl *N*-ıydroxymethylcarbamate, Sevin, and 1-ıaphthol, respectively, based on the adiotracer studies, responded as antici-ıated for 1-naphthol and its esters (*18*). The response of metabolites F and G ndicated the presence of a free phenolic ıydroxyl grouping on the carbamate nolecule. The yellow color with the luoborate reagent for metabolite G was imilar to that for 5-hydroxy-1-naphthyl *N*-methylcarbamate, while the orange olor with this reagent for F was similar o that for 4-hydroxy-1-naphthyl *N*-nethylcarbamate. The amount of netabolite C was always inadequate for neaningful tests. Metabolite B failed o respond to most of the chromogenic eagents, and where positive results were btained, the sensitivity was less than that or other metabolites. Metabolite D, vhen present in large amounts, gave a lue color with Gibbs' reagent, in the ame manner as did B subsequent but ıot prior to alkaline hydrolysis.

The 1,2-dihydro-1,2-dihydroxynaphthalene and its *N*-methylcarbamate were letected only with the fluoborate reagent nd in relatively large amounts. These naterials and metabolite B failed to luoresce after alkaline treatment. A urther possible indication of a similarity n structure for metabolite B and 1,2-lihydro - 2 - hydroxy - 1 - naphthyl *N*-nethylcarbamate was that they were imilar in chromatographic position, pproximately cochromatographing in he hexane-ether system and only being dequately resolved with the methylene hloride–acetonitrile system. Similar hromatographic results were obtained vith metabolite D and 1,2-dihydro-1,2-lihydroxynaphthalene. These findings night be interpreted as indicating some-vhat similar ring modifications with a reater phenolic nature for the metab-lites D and B than for 1,2-dihydro-1,2-lihydroxynaphthalene and its methyl-arbamate. Such a differentiation night result if the dihydrodihydroxy ype of structure was present in these netabolites at a site different than he 1,2-position. A 3,4-dihydro-3,4-dihydroxy-1-naphthyl *N*-methylcarbamate might respond in the manner reported for metabolite B, but no authentic compound was available for comparison.

Metabolism of *o*-Isopropoxyphenyl *N*-Methylcarbamate (Bayer 39007) by Liver Microsomes. Incubation of *o*-isopropoxyphenyl *N*-methylcarbamate-carbonyl-C^{14} with liver microsomes and $NADPH_2$ in a manner similar to that described for Sevin, and separation of the products on Florisil columns, yielded peaks comparable in position to II, III, and V from Sevin. Figure 3 shows the elution positions and solvents with Sevin. Peak II was found by TLC to be the original *N*-methylcarbamate. Peak V, eluting with methanol from Florisil, was not further investigated. Peak III was resolved by TLC into two materials. The R_f values for the 39007 metabolites with the 4:1 ether-hexane solvent system were almost identical to metabolites A, E, F, and H from Sevin. The efficiency of metabolism of 39007 was such that about 30% appeared as the metabolite in region E, 1 to 3% in region F, and most of the remaining radioactivity as the original carbamate (region H). Such a yield of metabolite E was considered sufficient to attempt isolation of the metabolite in milligram amounts.

Preparative scale microsome studies were conducted by using 30 flasks, each containing 1 mg. of nonlabeled and 100 μg. of C^{14}-labeled 39007. The ether-solubles from these reaction mixtures were chromatographed on Florisil to recover the peak III region, which was rechromatographed on the celite-hexane-acetonitrile column. About 2 mg. of a light yellow oil were obtained. This oil was immiscible with hexane and pentane but miscible with more polar organic solvents. Attempts to crystallize the product failed, and it was subjected directly to spectral and degradation analysis. This material consisted of a single component based on TLC and detection by chromogenic reagents or radioautography.

Degradation of this 39007 metabolite yielded *o*-isopropoxyphenol and formaldehyde. Injection of the metabolite into a gas chromatograph yielded an identical retention time to that obtained with *o*-isopropoxyphenol. The compound yielded a purple color with chromotropic acid as anticipated if under the acid conditions used with this reagent it decomposed to yield formaldehyde. When the metabolite was eluted from the Florisil column, the radioactivity and formaldehyde-yielding material were coincident. This metabolite was not *o*-isopropoxyphenyl *N*-methyl, *N*-hydroxy-carbamate or *o*-isopropoxyphenyl *N*-methoxycarbamate as it failed to co-chromatograph with these materials, and neither of these materials yielded a positive chromotropic acid test for formaldehyde. A sample of synthetic *o*-isopropoxyphenyl *N*-hydroxymethylcarbamate, prepared as previously described, cochromatographed with the radioactive metabolite on Florisil. This synthetic product yielded formaldehyde on decomposition and gave an infrared spectrum similar to the isolated metabolite of 39007. The additional absorption bands (2.9 to 3.0 and 9.8 to 9.9 microns) in the synthetic and microsome products (Figure 1, D and E) compared with 39007 (Figure 1 C) were consistent with those anticipated for a primary alcohol function as with *o*-isopropoxyphenyl *N*-hydroxymethylcarbamate.

The 39007 metabolite chromatographing on TLC in the position of the Sevin metabolite F might be a ring hydroxylation product, possibly in the para-position on analogy with Sevin metabolism, but no direct experimental evidence is available on this point.

Metabolism of Sevin by Insects. In preliminary studies involving topical application of Sevin labeled in the three different positions to *P. americana* and *M. domestica* adults, metabolites similar to those appearing with rat liver microsomes were formed and could be resolved on the Florisil column. The Sevin-carbonyl-C^{14} metabolites from houseflies appearing in peak III from Florisil were further resolved on TLC into metabolites E, F, and G. Topical application of Bayer 39007-carbonyl-C^{14} to these two species yielded metabolite fractions from Florisil comparable to the liver microsome metabolite fractions.

In a more complete investigation involving injection of 5 μg. of Sevin-carbonyl-C^{14} per roach (Table III), about 19% of the radioactivity was eliminated as carbon-14 dioxide in 24 hours. The remaining radioactive material not indicated in Table III was present as more polar derivatives or was excreted. Extractions of the insect residue with additional solvents indicated that complete recovery was achieved for Sevin and peaks III and IV. Resolution by TLC showed that peak III from the

Florisil was a mixture of metabolites E, F, and G, and that peak IV was a single component comparable to metabolite B from microsomes. When roaches were injected with either carbonyl-, methyl-, or naphthyl-labeled Sevin, TLC results comparable to those from rat liver microsomes were obtained (for radioautograms from microsome preparation, see Figure 4). The metabolites from the roaches appeared to be identical in respect to number, chromatographic position, and distribution of the labeled carbons to those from microsomes. The distribution of radioactivity among the various labeled metabolites at 2 hours after treatment (Table IV) indicated little hydrolysis of the administered compound with the major metabolites appearing in the water-soluble fraction. Results with the Sevin-carbonyl-C^{14} most clearly support this hypothesis. The predominant solvent-extractable metabolite appeared to be 1-naphthyl *N*-hydroxymethylcarbamate (metabolite E). The more extensive metabolism with Sevin-naphthyl-C^{14} than with the other two sites of labeling was probably related to more efficient distribution or more rapid enzymatic attack on the lower dose used with the ring-labeled material.

Cockroaches injected with 5 μg. of Bayer 39007-carbonyl-C^{14} were extracted with acetone 4 hours after injection and analyzed with the Florisil column. Three per cent of the injected dose remained as the original carbamate, while 12% appeared as the ether-extractable metabolite comparable to peak III from Sevin, and 7% as the fraction eluted with methanol. The remainder of the radioactivity was present as more polar products which were not extracted, or was given off as carbon-14 dioxide, which was not determined in this experiment. As with the microsome metabolites of this compound, peak III was resolved into two components chromatographing on TLC in the positions of the metabolites from this peak as formed by microsomes.

Table IV. Per Cent of Various Sevin Metabolites Two Hours after Injection of Cockroaches with Sevin-Carbonyl-C^{14}, Sevin-Methyl-C^{14}, and Sevin-Naphthyl-C^{14} Based on Extraction with Chloroform and Thin Layer Chromatography

Metabolites	Per Cent of Administered Radioactivity with Differently Labeled Samples Present as:[a] Carbonyl-C^{14}	Methyl-C^{14}	Naphthyl-C^{14}
Water-soluble	33.4	35.5	32.9
Ether-soluble			
A	3.9	2.9	5.0
B	2.5	2.1	2.9
C	...	...	0.7
D	...	...	1.0
E	5.9	5.7	7.3
F	1.7	1.8	2.5
G	1.1	1.0	1.5
H (Sevin)	47.8	48.0	36.7
I	...	...	5.6
Carbon dioxide	1.0	0.3	0.0
Total % recovery	97.3	97.3	96.1

[a] Sevin-carbonyl-C^{14} and Sevin-methyl-C^{14} injected at 5 μg. per insect and Sevin-naphthyl-C^{14} at 2 μg. per insect.

Sevin Metabolites in Milk. Oral administration of Sevin-carbonyl-C^{14} at 1.34 mg. per kg. to a goat resulted in excretion of 47% of the radioactivity in the urine during the 96-hour experiment. The cumulative percentages of the administered radioactivity detected in the urine at intermediate times after treatment were as follows: 7.4 at 2 hours, 24 at 4 hours, 36 at 8 hours, 41 at 12 hours, and 45 at 24 hours. Evaporation onto Florisil of unextracted urine samples collected at 2, 4, 8, and 12 hours after treatment resulted in three fractions when the columns were developed. The first two fractions were in the positions indicated as peaks IV and V (see Figure 3 for peak positions using metabolites formed by microsomes) and the third fraction was eluted by a final water wash after the methanol elutriant. The fraction in the peak IV region accounted for about 5 to 10% of the radioactivity, and the methanol eluted about one half the amount recovered in the final water wash of the column. Extraction of these Sevin urine samples with chloroform resulted in recovery of a single metabolite which was identical to metabolite B on both the Florisil column and TLC.

The total p.p.m. Sevin equivalents reached a peak in the milk of about 0.9 p.p.m. at 8 hours but diminished thereafter so that only 0.003 p.p.m. was detected at 60 hours (Table V). Most of this radioactivity appeared in the water-soluble (not extracted by organic solvents) and milk solids fractions, and no method was found for separating the metabolites from these fractions for further investigation. The organosoluble or solvent-extractable fractions, which reached a peak level of about 0.3 p.p.m. at 4 hours (Table V), were subjected to chromatography on the Florisil column (Table VI). Very little Sevin (maximum 0.004 p.p.m.) was detected, and then only in the early samples. One organosoluble metabolite eluted in the region of peak IV from microsomes and was assumed therefore to be the same as the microsome product designated as metabolite B. A metabolite not detected in any of the other biological systems described, and designated as "metabolite X," appeared in relatively small amounts in the 8- to 20-hour milk samples. This metabolite eluted from the Florisil column between 1-naphthol and Sevin, being partially resolved from the former and completely from the latter. Since carbonyl-labeled material was used in the study, the inability to

Table V. Residues in Milk from Oral Administration of 1.34 Mg. per Kg. of Sevin-Carbonyl-C^{14} to a Goat

P.P.M. Sevin-C^{14} equivalents in whole milk, and organosoluble ($CHCl_3$–CH_3CN), water-soluble, and milk solids fractions

Hours after Treatment	P.P.M. Sevin-Carbonyl-C^{14} Equivalents in: Whole milk	Water-soluble	Organo-soluble	Milk solids
2	0.297	0.064	0.209	0.024
4	0.778	0.188	0.295	0.195
8	0.928	0.568	0.204	0.156
12	0.766	0.398	0.101	0.267
16	0.472	0.242	0.095	0.135
20	0.322	0.132	0.018	0.172
24	0.199	0.050	0	0.140
28	0.147	0.035	0	0.112
32	0.107	0	0	0.107
36	0.071	0	0	0.071
48	0.006	0	0	0.006
60	0.003	0	0	0.003
72–96	0	0	0	0

Table VI. Residues in Milk from Oral Administration of 1.34 Mg. per Kg. of Sevin-Carbonyl-C^{14} to a Goat

Resolution of organosoluble ($CHCl_3$–CH_3CN) metabolites by chromatography on Florisil

Hours after Treatment	P.P.M. Sevin-Carbonyl-C^{14} Equivalents as: Sevin	Peak IV	Metabolite "X"	Peak V
2	0.004	0.075	0	0.102
4	0.003	0.056	0	0.224
8	0.003	0.007	0.006	0.085
12	0	0	0.007	0.045
16	0	0	0.027	0.025
20	0	0	0.008	0.011
24–96	0	0	0	0

'able VII. Total Sevin-Carbonyl-C^{14} and Bayer 39007-Carbonyl-C^{14} Equivalents Recovered from Injected Bean and Cotton Plants Based on Acetone Extraction and Chromatography on Florisil

Days after Treatment	Per Cent Recovery of Administered Radioactivity					
	Beans				Cotton	
	Sevin		Bayer 39007		Bayer 39007	
	Total C^{14}	Orig. cmpd.	Total C^{14}	Orig. cmpd.	Total C^{14}	Orig. cmpd.
1	97.8	92.7	89.6	86.9	98.2	75.2
2	83.3	76.1	80.8	70.6	84.8	59.9
4	98.4	74.5	88.8	62.3	94.5	39.1
7	87.2	59.1	93.4	42.6	88.4	17.7
14	45.4	6.9	91.3	17.7	93.5	7.5
28	55.4	5.7	77.5	7.8	86.0	1.7

ompletely resolve this metabolite from -naphthol did not pose a problem. The major organosoluble components were eluted from the Florisil with methnol, but some further radioactivity ould be washed from the column with ater following the methanol elution. his additional component which could e eluted with water is not considered in ie data presented in Table VI. The ifference between the summation of the actions in Table VI compared with ie total p.p.m. Sevin equivalents in ie chloroform-acetonitrile extract indiated in Table V probably resulted from iis additional component eluting with ater. No peak III (metabolites E, F, nd G) appeared in the milk despite very sensitive system for its detection present.

Metabolism of Sevin and Bayer 39007 y Plants. The plant metabolites of evin-C^{14} and Bayer 39007-carbonyl-C^{14} ere separated only into the original ompound and a water-soluble or nonrganic solvent-extractable fraction. oth of these fractions were recovered by omogenizing the whole plants in cetone and chromatographing the aceone-soluble materials on Florisil. When ie plants were extracted with acetone, ie residue was re-extracted with chloroirm, and these combined were solvents ashed with water, only original carbamate was recovered in the organoluble portion. The material remaining i the water, when separately subjected o chromatography on Florisil, eluted ith methanol in the region of peak V. his latter procedure therefore separated ie original carbamate from its waterluble metabolites without the necessity or chromatography. No metabolites omparable to peaks III or IV from evin or III from 39007 were recovered y chromatography on Florisil.

The percentage recovery of the inected radioactivity with beans and otton remained high through the 28-ay experiment, despite the fact that the riginal carbamate had largely disppeared during this period and the ompounds were labeled in the carbonyl osition (Table VII). The more waterluble Bayer 39007 yielded more consistent results in this respect than did the Sevin, where limited solubility may have resulted in greater treatment variation. The carbamates were therefore converted into water-soluble metabolite(s) which were quite stable within the plants. Similar results were obtained on injection of Sevin-methyl-C^{14} and Sevin-naphthyl-C^{14} into beans, indicating that the water-soluble metabolite(s) may have been carbamates. Limited studies designed to resolve the water-soluble metabolite(s) of Sevin and Bayer 39007 into more than one component were unsuccessful.

Biological Activity of Metabolites of Sevin and Bayer 39007. Limited studies were made on the metabolites of Sevin and Bayer 39007 as recovered from microsome preparations. Peak III (a mixture of metabolites E, F, and G) and peak IV (metabolite B) derivatives of Sevin and the mixture of two metabolites of 39007 which appeared in the peak III region were separated from other radioactive materials on the Florisil column and assayed for anticholinesterase activity in vitro with fly head homogenates. The radioactive materials eluting with methanol were also assayed. The molar levels of carbamate for 50% inhibition were $7.0 \times 10^{-8}M$ for Sevin and $2.4 \times 10^{-8}M$ for Bayer 39007. Levels for 50% inhibition from the metabolites were $5.2 \times 10^{-7}M$ with peak III of Sevin, $5.6 \times 10^{-7}M$ with peak IV of Sevin, and $5.4 \times 10^{-7}M$ with peak III of 39007. The metabolites eluting with methanol were less active (greater than $8 \times 10^{-7}M$ for 50% inhibition) as anticholinesterase agents than the other materials assayed. The peak III and probably also the peak IV fractions from both Sevin and 39007 were mixtures of metabolites as discussed earlier.

Sevin metabolites B, E, and F as separated by TLC were compared in toxicity to Sevin with female houseflies. The flies were treated with piperonyl butoxide at 500 μg. per gram, and then immediately afterward with Sevin or its radioactive metabolites. At 4 hours, the mortality from Sevin at 12 μg. per gram was 80%. Metabolites B, E, and F at 60 μg. per gram yielded per cent mortality figures of 40, 20, and 30, respectively. Thus with a high level of synergist, the metabolites were much less than one fifth as toxic as Sevin.

The biological activity of synthetic 1-naphthyl *N*-hydroxymethylcarbamate was compared with that of Sevin. Carbamate levels for 50% cholinesterase inhibition were $7.0 \times 10^{-8}M$ with Sevin and $3.0 \times 10^{-5}M$ with the hydroxymethyl derivative. Topical LD_{50} values at 4 hours for female flies pretreated with 500 μg. per gram of piperonyl butoxide were 7.8 μg. per gram for Sevin and greater than 10,000 μg. per gram for the hydroxymethyl derivative. Mouse intraperitoneal LD_{50} values at 24 hours were 35 to 42 mg. per kg. for Sevin and 630 to 780 mg. per kg. for 1-naphthyl *N*-hydroxymethylcarbamate.

Discussion

The ether- or chloroform-extractable metabolites of Sevin as recovered from rat liver microsomes appeared to be identical to those recovered from two insect species. A minor attack on the molecule involved hydrolysis to yield 1-naphthol. The methyl group was modified in one metabolite, probably to yield 1-naphthyl *N*-hydroxymethylcarbamate. Ring hydroxylation occurred forming 4-hydroxy-1-naphthyl *N*-methylcarbamate and trace amounts of 5 - hydroxy - 1 - naphthyl *N*-methylcarbamate. Another metabolite may have been an *N*-methylcarbamate with a dihydro-dihydroxy function on adjacent carbons in the ring, although only limited evidence is available on this material. Certain metabolites appeared to be hydrolysis products of carbamates with those modified ring structures. The metabolism of the related compounds, naphthalene, dihydronaphthalene, and 1-naphthol, has been extensively studied in insects, mammals, and liver microsomes (*1, 3–5, 19, 21*). A metabolic pathway proposed for hydroxylation of naphthalene (*5*) is particularly intriguing in respect to the nature of the products recovered from Sevin. By combining the pathway proposed for naphthalene with the available evidence on the nature of the Sevin metabolites, a pathway such as indicated in Figure 5 can be obtained. Although this pathway appears to be consistent with the known properties for the metabolites most extensively studied, it is no more than a working hypothesis for more critical studies on the detoxication of Sevin. Very little evidence is available on the chemical nature of metabolite B, and almost none on the nature of the water-soluble fractions, which are possibly conjugates of carbamates and their hydrolysis products. These conjugates would be expected to consist of the

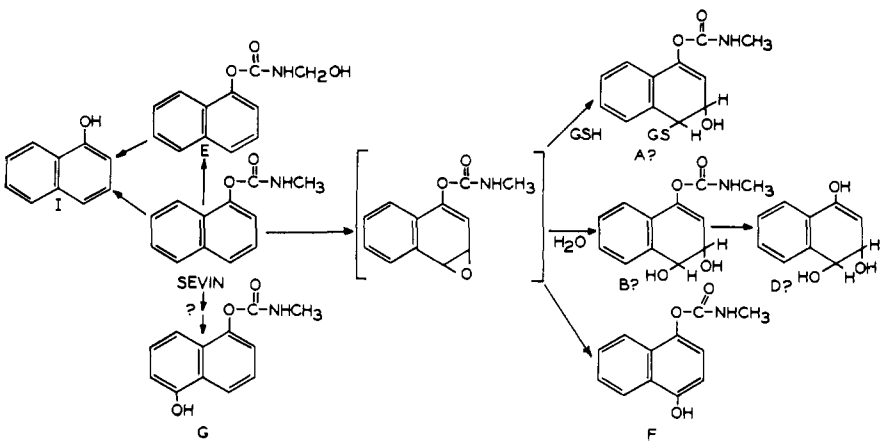

Figure 5. Hypothetical pathway for Sevin metabolism by rat liver microsomes, houseflies, and cockroaches

Based on tentative identification of certain metabolites (E, F, G, and I) and interpretation from published studies (see text) on naphthalene metabolic pathway (epoxide intermediate and unidentified metabolites A, B, and D, where GSH indicates glutathione); pathway does not include possible conversion of naphthol derivatives to glucuronides, glucosides, and sulfates

glucosiduronic acids, sulfates, mercapturic acids, and *N*-acetylmercapturic acids, and, in certain cases also the glucosides (*2, 4–7, 11, 17, 21, 23, 25*). Hydrolysis, methyl hydroxylation and ring hydroxylation, and conjugation before or after hydrolysis of the carbamyl grouping are known pathways for metabolism of biologically active carbamates (*8, 9*). The biological significance of such detoxication mechanisms in insects in relation to species specificity, resistance mechanisms in selected strains, and action of synergists has also been discussed in these reviews.

It is of particular interest to consider the present observations in relation to the residues encountered with the use of Sevin. The principal method utilized to analyze for this insecticide involves saponification and reaction of the 1-naphthol with *p*-nitrobenzenediazonium fluoborate. One of the principal metabolites, which appeared in low levels in milk from a Sevin-treated goat, failed to respond to this chromogenic reagent. Other metabolites which did respond might be lost during clean-up procedures. Major metabolites from both plants and mammals were not extractable into organic solvents and thus would escape detection, and their chemical nature is not known. Where sufficient amounts of organosoluble metabolites were available for limited bioassay, they appeared to be less active than Sevin per se. Further information on the chemical nature and toxicity of the carbamate metabolites of Sevin would be helpful in interpreting the residue levels reported in relation to the residue hazard.

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