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CARBARYL METABOLITES

Metabolites of Carbaryl (1-Naphthyl Methylcarbamate) in Mammals and Enzymatic Systems for Their Formation

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Ether-extractable carbaryl (1-naphthyl methylcarbamate) metabolites found in the urine of treated rabbits and formed by enzyme preparations from rat, mouse, and rabbit liver are tentatively identified as follows: 1-naphthyl *N*-hydroxymethylcarbamate; 4-hydroxy-1-naphthyl methylcarbamate; 5-hydroxy-1-naphthyl methylcarbamate; 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate; 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene; and 1-naphthol. Additional unidentified metabolites are also present in ether and butanol extracts of the urine. Each of the ether-extractable metabolites formed by liver enzymes is of reduced biological activity compared with carbaryl. Optimum conditions are given for metabolism of carbaryl by rat liver microsomes plus soluble fractions; inhibition of the metabolism system by five insecticide synergists is demonstrated. Hydroxylated carbaryl metabolites are probably conjugated, in part, as glucuronides and ethereal sulfates, based on in vitro studies with conjugation systems.

METABOLISM of carbaryl (1-naphthyl methylcarbamate) by mammals includes both hydrolytic and nonhydrolytic pathways. Plasma albumin acts as an enzyme in effecting carbaryl hydrolysis (*5*). Hydroxylation of the ring at the 4- and 5-positions and of the *N*-methyl group results from incubation of carbaryl with rat liver microsomes fortified with reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) (*7, 8*). An additional metabolite formed by microsomes in vitro and found in the milk and urine of a treated goat is assumed to be 3,4-dihydro-3,4-dihydroxy-1-naphthyl methylcarbamate based on analogy with the metabolic pathway for naphthalene (*7*). 1-Naphthol derived from carbaryl metabolism is assumed to be conjugated and excreted in the urine as 1-naphthyl glucuronide by humans, rats, and guinea pigs (*1, 4, 25*), and 1-naphthyl sulfate is known to be a carbaryl metabolite in cow urine (*25*). One half to two thirds of the radiocarbon from carbaryl-carbonyl-C^{14} administered to rats and a goat is eliminated in the urine (*7, 15*), indicating that no more than half of the dose is hydrolyzed under these conditions. Several conjugates of carbaryl metabolites, as formed in isolated systems and as excreted in the urine of treated animals, were recently identified by Knaak *et al.* (*14*) in a report which appeared after the present investigation was accepted for publication.

The present investigation supplements the available information on the nature of carbaryl metabolites (*7*) and gives a more complete picture of the nonconjugated metabolites excreted in urine and, particularly, the enzymatic systems for their formation.

Methods and Materials

Chemicals and Apparatus. Carbaryl-naphthyl-1-C^{14} was synthesized by reaction of 1-naphthol-1-C^{14} (Nuclear-Chicago Corp., Des Plaines, Ill.) with a slight excess of methyl isocyanate in a sealed ampoule in the dark for 48 hours at 25° C. The excess methyl isocyanate was evaporated and the product was purified on a Florisil column (*16*). The radiochemical purity of each batch was greater than 99.9%, as determined by cochromatography with authentic carbaryl (analytical purity, Union Carbide Chemicals Co., New York, N. Y.) on Silica Gel G chromatoplates. The specific activity of carbaryl-naphthyl-1-C^{14} was adjusted to 1.0 mc. per mmole (approximately 2500 c.p.m. per μg.).

Carbaryl and its metabolites were resolved by chromatography, detected by radioautography or chromogenic agents, and quantitatively determined by scintillation counting. Florisil (60- to 100-mesh) for column chromatography was obtained from the Floridin Co., Tallahassee, Fla., and was used without activation (*16*). Thin-layer chromatography (TLC) utilized Silica Gel G or aluminum oxide G (Brinkman Instruments, Inc., Great Neck, N. Y.) chromatoplates. To detect fluorescent regions corresponding to certain carbaryl metabolites, the silica gel plates were examined under long-wavelength ultraviolet light (Model SL 3660 Mineralight, Ultra-Violet Products Inc., South Pasadena, Calif.). Carbaryl, 1-naphthol, and certain metabolites were detected by spraying the chromatoplate with 15% aqueous potassium hydroxide followed by 0.1% Gibbs' (*N*,2,6-trichloro-*p*-benzoquinoneimine) reagent (*7, 16*). Carbamates were detected by spraying with 1% ninhydrin in pyridine followed by a 30-minute period of heating at 100° C. (*7*); pyridine without ninhydrin was used as a control. Radioactive compounds on the thin-layer chromatograms were detected by radioautography using medical "no screen"-type x-ray film (Eastman Kodak Co., Rochester, N. Y.).

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Quantitative radioactive measurements were made with a Packard Tri-Carb Series 314 E liquid scintillation spectrometer, using 15 ml. of 0.55% 2,5-diphenyloxazole in toluene–ethylene glycol monomethyl ether mixture (2 to 1) in each vial.

Cofactors for the various enzymatic reactions were obtained from the following sources: reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), nicotinamide-adenine dinucleotide (NAD), and adenosine-5-mononicotinate from Nutritional Biochemicals Corp., Cleveland, Ohio; nicotinamide-adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide ($NADH_2$), NAD analogs modified on the nicotinamide moiety (3-acetylpyridine-NAD, 3-pyridinealdehyde-NAD, thionicotinamide-NAD), the adenine moiety (deamino-NAD), or both the nicotinamide and adenine moieties (3 - acetylpyridine - deamino-NAD), nicotinamide mononucleotide, 1-methyl nicotinamide iodide, adenosine triphosphate, and uridine diphosphoglucuronic acid from the California Corp. for Biochemical Research, Los Angeles, Calif. Potential inhibitors for the metabolism were from the following sources: α - [2 - (2 - butoxyethoxy)-ethoxy] - 4,5 - methylenedioxy - 2-propyltoluene (piperonyl butoxide) from U. S. Industrial Chemicals Co., New York, N. Y.; β-diethylaminoethyl diphenyl propyl acetate hydrochloride (SKF-525A) from Smith, Kline, and French Co., Philadelphia, Pa.; *N*-(2-ethylhexyl) - 5 - norbornene - 2,3-dicarboximide (MGK 264) from McLaughlin, Gormley, King Co., Minneapolis, Minn.; 2-(3,5-dichloro-2-biphenyloxy)ethyldiethylamine (Lilly 18947) and 2-(2,4-dichloro-1-naphthoxy)ethyldiethylamine from H. T. Gordon, Division of Entomology and Acarology, University of California, Berkeley, Calif.

Melting points were determined with a Fisher-Johns melting point apparatus (Fisher Scientific Co., Chicago, Ill.) on individual crystals placed between two microscope cover slips; they were not corrected. Infrared spectra were obtained using potassium bromide pellets and a Model 4-55 Baird double-beam infrared spectrometer with sodium chloride optics (Baird-Atomic, Inc., Cambridge, Mass.).

Analysis of Urine from Rabbits Treated with Carbaryl or 1-Naphthol. Adult rabbits (New Zealand White strain, male and female, 1.5 to 2.8 kg.) were treated subcutaneously with 5 mmoles of carbaryl (1.008 grams) or 1-naphthol (0.721 gram) in 2 ml. of corn oil each day for 10 days; this carbaryl or 1-naphthol dose gave no toxic symptoms. The rabbits were kept in metabolism cages, allowing separate collection of urine and feces, and were supplied with rabbit pellets (National Pro 17, National Alfalfa Dehydrating and Milling Co., Kansas City, Mo.) and water *ad libitum*. Urine was collected daily throughout the treatment period and for 2 days thereafter. Urine was also collected from untreated rabbits and rabbits injected subcutaneously with only 2 ml. of corn oil per day. The urine, stored at −15° C. until analyzed, was extracted three times with equal volumes of ether, and the combined ether extracts were dried with sodium sulfate and evaporated under reduced pressure. Subsequently, the urine was similarly extracted three times with equal volumes of 1-butanol. The efficiency of extraction is not known, although little, if any, material responding to the potassium hydroxide–Gibbs' reagent (described below) was recovered by an additional extraction with either ether or 1-butanol. The average daily weight of the ether and 1-butanol extracts, after removal of solvents, was determined for the first 3 days following initiation of the treatment schedule.

Each ether-extractable fraction was analyzed by two-dimensional TLC to determine which spots were uniquely attributable to metabolites derived from carbaryl or 1-naphthol. A portion containing 1 mg. of solids of each ether extract was spotted for these chromatograms. The 20 × 20 cm. plates were developed in the first direction with ether-hexane (9 to 1) and then in the second direction with chloroform-acetonitrile (2 to 1). The solvent fronts were allowed just to reach the edge of the TLC plates. Chromogenic agents used to detect these metabolites were as follows: 15% aqueous potassium hydroxide to yield yellowish to brown spots; Gibbs' reagent to give spots of widely varying colors; 15% aqueous potassium hydroxide followed by Gibbs' reagent to give an even wider variety of colors; pyridine followed by 30 minutes at 100° C. to yield yellow to dark brown spots; and 1% ninhydrin in pyridine, with a 30-minute color development period at 100° C., to yield red spots from the carbamates. The number and position of spots detected by each reagent were determined for extracts made at various times after initiation of the treatment schedule; the values obtained were averaged. The ether extracts of urine were fortified with carbaryl-naphthyl-1-C^{14} metabolites from liver microsomes (prepared as described below) to determine, by cochromatography and radioautography, which metabolites formed by microsomes were identical to ones present in the urine.

Compounds present in the 1-butanol extracts of urine were poorly resolved by TLC. Four solvent systems gave promising resolution when used in one direction: 1 - butanol – acetic acid – water (2:1:1); 1 - butanol – ethanol – water (17:3:20); acetone – ethyl acetate–ethanol (2:1:1); and 1-butanol–1-propanol-0.1*N* ammonium hydroxide (2:1:1). However, when these solvents were used in combination for two-dimensional TLC, there invariably were streaking and overlapping of the spots. Two-dimensional chromatograms of the 1-butanol extracts were very carefully inspected to estimate the number of spots present.

Florisil column chromatography was used for purification and isolation of metabolites obtained by ether extraction of the urine from carbaryl-treated rabbits. Approximately 30 grams of Florisil were slurried in hexane and packed to yield a 2 × 30 cm. column. The ether extract was evaporated to dryness under reduced pressure onto about 1 gram of Florisil, which was then placed on top of the packed column. Elution was accomplished with the following solvent sequence: 400 ml. each of 1 to 1, 2 to 1, 3 to 1, and 4 to 1 ether-hexane; 600 ml. of ether; 200 ml. of methanol. Twenty-milliliter fractions were collected at a flow rate of approximately 8 ml. per minute. Metabolites in eluted fractions were detected by spotting 50-μl. aliquots on filter paper and using the potassium hydroxide–Gibbs' reagent for spot development. Several fractions in each metabolite region were spotted on TLC plates and cochromatographed with carbaryl-naphthyl-1-C^{14} metabolites from liver microsomes using the 9 to 1 ether-hexane solvent mixture. Fractions containing an unlabeled metabolite from urine which cochromatographed in one direction with a labeled microsome metabolite were rechecked by two-dimensional TLC, and the appropriate fractions were then pooled and rechromatographed on Florisil. A third Florisil purification was sometimes necessary for clear separation of metabolites.

The results obtained are shown in Table I and Figure 1.

Isolation of Metabolites from Carbaryl-Liver Microsome System. Carbaryl metabolites, for characterization studies, were also obtained from the carbaryl–rat liver microsome system, described in detail below, but modified in the amount of certain incubation constituents for use with a higher substrate level. Each flask contained 0.25 mg. of carbaryl, the microsome fraction equivalent to 3 ml. of 20% rat liver homogenate, 1.5 ml. of the soluble fraction, and 2.0 μmoles of NAD. After 4 hours of incubation in air with shaking at 37° C., the incubation mixtures were extracted with ether. The ether was dried with anhydrous sodium sulfate, the solvent removed under reduced pressure, and the residue spotted at 1-cm. intervals near the bottom of silica gel G (0.5-mm. thickness) chromatoplates. Chromatograms were developed for 18 cm. with 1 to 1 ether-hexane mixture and a strip, 1 cm. along one edge, was sprayed with the potassium hydroxide–Gibbs' reagent to locate the metabolite spots. Fluorescent areas due to certain carbaryl metabolites were observed under long-wave ultraviolet light. Regions of the plates containing a particular metabolite were scraped into separate flasks and extracted with ether and then acetone. The two extracts for each metabolite were combined, dried with anhydrous sodium sulfate, and evaporated to approximately 1 ml. under reduced pressure. Those solutions which might contain a pure metabolite, based on a single spot resulting from TLC analysis and potassium hydroxide–Gibbs' reagent, were stored at 2° C., while the impure solutions were rechromatographed until only a single spot was detected by TLC analysis using this chromogenic reagent. Certain metabolites, designated

Table I. Analysis of Urine of Rabbits Treated Daily by Subcutaneous Injection of Carbaryl (5 Mmoles) or 1-Naphthol (5 Mmoles) in Corn Oil, or Corn Oil Alone, as Compared with Untreated (Control) Rabbits

Material Analyzed	Amount Resulting from Injection of: Nothing (control)	Corn Oil: Alone	Corn Oil: 1-Naphthol	Corn Oil: Carbaryl
	Weight of Extractives, Mg./Day			
Ether extract	41	50	81	105
1-Butanol extract	1200	1300	2100	2300
	Number of TLC Spots Detected, with Various Chromogenic Agents			
Ether extract				
Gibbs'	26	26	28	37
KOH	21	21	21	34
KOH plus Gibbs'	19	19	19	30
Ninhydrin (1%) in pyridine	19	19	19	28
Pyridine	19	19	19	28
1-Butanol extract				
Gibbs'	15	15	17	21
KOH	11	11	12	16
KOH plus Gibbs'	18	18	20	25

as D, E, F, and G, could not be completely resolved from contaminating fluorescent bands.

Biological Activity of Carbaryl Metabolites. Bioassays were made on carbaryl and certain of its ether-extractable metabolites as recovered from rabbit urine and the rat liver–enzyme system. Three-day-old adult female houseflies (*Musca domestica* L., 1948 C.S.M.A. susceptible strain, 22-mg. average weight) were treated topically on the ventrum of the abdomen with 1 μl. of acetone containing varying amounts of the test compound and on the dorsum of the abdomen with 1 μl. of acetone containing 10 μg. of piperonyl butoxide. Mortality from the synergized carbaryl or metabolites was recorded after 24 hours.

Anticholinesterase activity of carbaryl and its metabolites, as formed in vitro by rat liver microsomes, was determined with a modification of the method of Crosby, Leitis, and Winterlin (*6*). The modifications consisted of slurrying the aluminum oxide G with deionized water (1 to 2, w./v.), instead of 50% aqueous methanol, and increasing the drying time for the chromatoplates to 75 minutes. Solutions of carbaryl and/or its metabolites were spotted 3 cm. from either edge near one corner of a chromatoplate. The two-dimensional chromatograms were first developed for 17 cm. with 9 to 1 ether-hexane mixture and then, in the other direction, for 17 cm. with 2 to 1 chloroform-acetonitrile mixture. The chromatograms were run in duplicate: One was analyzed for cholinesterase inhibition; the other was radioautographed and the amount of each metabolite was determined by scraping the gel regions, corresponding to dark spots on the radioautogram, into scintillation vials and counting. The minimum amount of resolved compound necessary to give a definite spot due to cholinesterase inhibition was correlated with the radioactivity measurement to derive the micrograms of carbaryl equivalents needed to produce cholinesterase inhibition.

Preparation of Liver Fractions. Male albino rats (170 to 190 grams, Rolfsmeyer Farms, Madison, Wis.) were killed by cerebral concussion and the livers removed as quickly as possible and immersed in 0.25*M* sucrose at 0° C. After chilling for 3 minutes, the livers were blotted free of excess liquid, weighed, and homogenized at 20% (w./v.) in 0.25*M* sucrose for 1 minute with a chilled Potter-Elvehjem type homogenizer utilizing a Teflon pestle. The mixture was centrifuged at 0° to 2° C. for 30 minutes at 15,000 g to sediment the debris (undisrupted cells, nuclei, mitochondria, erythrocytes, etc.). Centrifugation of the supernatant liquid at 95,000 g for 30 minutes at 2° C. sedimented the microsome pellet, from which the soluble fraction was decanted. The microsomal pellet was washed twice by resuspension in 0.25*M* sucrose, followed by centrifugation at 95,000 g for 30 minutes at 2° C. After decantation of the last supernatant liquid, the microsomal pellet was frozen and stored at −15° C.; the activity of these pellets for metabolism of carbaryl was undiminished on frozen storage for up to one week before use. The individual pellets were resuspended in a volume of 0.25*M* sucrose such that the mixture was equivalent in concentration to the microsomes from a 40% liver homogenate. In certain experiments, microsomes prepared in the same manner from mice and rabbits were utilized.

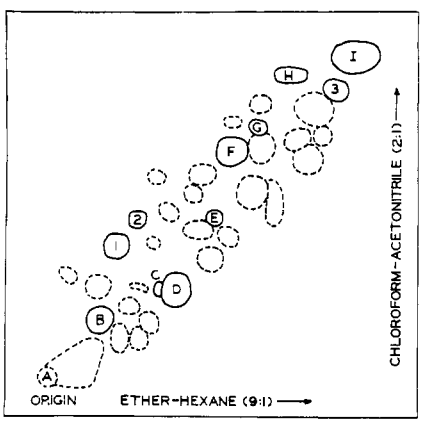

Figure 1. Carbaryl metabolites present in ether extracts of urine from carbaryl-treated rabbits as resolved by thin-layer chromatography

Metabolism of Carbaryl by Rat Liver Microsomes. For a typical experiment, 40 μg. of carbaryl-naphthyl-1-C^{14} (100,000 c.p.m.) were added to the bottom of a 25-ml. Erlenmeyer flask in 100 μl. of ether and the solvent was allowed to evaporate at room temperature. Then, the following materials were added to the flask: 100 μmoles of sodium phosphate buffer (pH 7.3, in 0.5 ml. of distilled water); 2 μmoles of pyridine nucleotide cofactor; 16 μmoles of nicotinamide; 2 μmoles of barium acetate; 0.5 ml. of liver microsomes (equivalent to 200 mg. of liver) in 0.25*M* sucrose and/or 0.5 ml. of the soluble fraction from the liver (equivalent to 100 mg. of liver); and 0.5 ml. of distilled water or 0.5 ml. of distilled water plus 0.5 ml. of 0.25*M* sucrose to make a volume of 2.0 ml. The flasks were shaken aerobically at 37° C. for 4 hours on a Dubnoff metabolic shaking incubator. This standard incubation mixture and these reaction conditions were used in all studies unless specifically noted otherwise; usually, no more than a single constituent was varied at one time.

After incubation, the mixtures were immediately either extracted or frozen. The frozen samples were stored at −15° C. for up to 24 hours before extraction. For extraction, each incubation mixture was poured into a 15-ml. centrifuge tube and the flask was rinsed separately with 3 ml. of distilled water and 5 ml. of ether, both being added to the tube. The aqueous phase was extracted three times with 5-ml. portions of ether; preliminary tests with labeled metabolite mixtures indicated that little, if any, additional radiocarbon was recovered in the third of these extractions. The combined ether extract was dried with anhydrous sodium sulfate and evaporated under reduced pressure, in a rotary evaporator, to approximately 0.5 ml., after which it was quantitatively transferred with ether rinses to a graduated centrifuge tube and reduced to 0.2 ml. with a stream of nitrogen. One hundred microliters of the ether extract were spotted on a Silica Gel G chromatoplate, of 0.25-mm. gel thickness, and the remaining fraction was taken up in 15 ml. of liquid scintillation mixture for counting. The thin-layer chromatogram was developed 18 cm. with ether-hexane (9 to 1). Radioactive regions on the plates, corresponding to darkened areas on the radioautogram, were scraped into 20-ml. scintillation vials for direct measurement of radioactivity. It was determined, in separate experiments, that this procedure for radioassay of resolved metabolites by counting the scraped regions gave an average C^{14}-recovery of 95% of that originally spotted on the plates. The ether-extractable metabolites were designated as A to I, in accordance with Dorough and Casida (*7*).

The percentage of the initial radiocarbon appearing in the different frac-

tions was derived from the total counts per minute for these fractions in the following way: A 0.2-ml. aliquot from the 5-ml. aqueous phase remaining after ether extraction was counted and the result multiplied by 25 for the total counts per minute of metabolites in the aqueous fraction; the ether remaining after 100 μl. was spotted on the TLC plate was counted and this figure was added to the total carbon-14 recovered from all radioactive regions on the plate, after correction for recovery from TLC, to obtain the total ether-extractable counts per minute; the per cent of the total carbon-14 present in each ether-extractable metabolite, as resolved by TLC, was multiplied by the total ether-extractable counts per minute to derive the actual counts per minute of each metabolite.

The ether-extractable carbaryl metabolites, as recovered from microsome plus $NADPH_2$ reactions, were incubated with selected conjugation systems to ascertain the ease of conjugation of the various hydroxylated derivatives. In the first study, metabolites having hydroxyl groupings were formed with the microsome plus $NADPH_2$ system and recovered as a metabolite mixture by extraction into ether; then the ether was evaporated and the labeled products were incubated with the conjugation system. The systems used were as follows: glutathione conjugation based on Booth, Boyland, and Sims (*2*); glucuronide conjugation based on Dutton (*9*); sulfate conjugation based on Roy (*23*). Each incubation flask contained approximately 40 μg. of ether-extractable carbaryl-naphthyl-1-C^{14} metabolites, 125 μmoles of sucrose, 100 μmoles of KH_2PO_4-Na_2HPO_4 buffer (pH 7.3), tissue fractions (when present) equivalent to 200 mg. of liver, and distilled water to make 3.0 ml. Additional constituents were: 10 μmoles of reduced glutathione for the glutathione conjugation system; 0.2 μmole of uridine diphosphoglucuronic acid for the glucuronide conjugation system; 4 μmoles of adenosine triphosphate, 1.5 μmoles of magnesium sulfate, 9 μmoles of potassium sulfate, and 90 μmoles of KH_2PO_4 for the sulfate conjugation system. The final pH of all incubation mixtures was adjusted to 7.3. In a separate study, these incubation mixtures utilized carbaryl-naphthyl-1-C^{14} as the substrate and also included 2 μmoles of $NADPH_2$, 2 μmoles of barium acetate and 16 μmoles of nicotinamide to determine whether conjugation of metabolites with hydroxyl groupings could be effected as they were formed.

In the early exploratory work, microsomes from mice, rats, and rabbits (both young and old of each sex) were utilized. However, in all subsequent studies, adult male rats were used because of their availability and the ease with which sufficient quantities of microsomes could be obtained from them, and because comparable results were obtained with the three species of animals.

The results obtained are given in Tables II to V.

Results

Analysis of Urine from Rabbits Treated with Carbaryl or 1-Naphthol. Daily subcutaneous administration to rabbits of 5 mmoles of either carbaryl or 1-naphthol (in corn oil) resulted in excretion in the urine of materials not present in the urine from untreated rabbits or rabbits treated with corn oil alone (Table I and Figure 1). About 31 mg. per day of additional ether-extractable material resulted from 1-naphthol administration, and 55 mg. per day from carbaryl administration. The additional weight recovered on subsequent 1-butanol extraction of the urine accounted for almost all of the weight of the administered compound. Much of this weight, however, might have resulted from natural materials used in formation of conjugates of the metabolites. The weight of metabolites remaining in the water fraction is not known.

Each of the ether-extractable metabolites of carbaryl produced by rat liver microsomes (metabolites A to I) was also present in ether extracts of urine from carbaryl-treated rabbits based on cochromatography studies. Three additional in vivo metabolites (1, 2, and 3) were detected in urine of carbaryl-treated rabbits, but not in urine from 1-naphthol- or corn oil–treated rabbits or in untreated rabbits; a fourth additional spot, appearing only with the carbaryl-treated rabbits, was detected only by the potassium hydroxide spray but not by any of the other chromogenic agents. All other spots, indicated by dashed circles, were detected by one or more of the chromogenic agents and were present in the urine of untreated rabbits, rabbits treated with corn oil, or corn oil containing 1-naphthol as well as corn oil containing carbaryl (Figure 1). Most of the spots noted in Table I as being detected with KOH alone, pyridine alone, or ninhydrin (1%) in pyridine were yellow to tan. Such spots were difficult to distinguish when KOH–Gibbs' reagent was used, because the TLC plate had a yellow background color. This fact accounts for the detection of fewer spots from the ether-extractables with this treatment than with either of these reagents separately. The ninhydrin reagent gave the characteristic pink to red color indicating an amine precursor only with the carbaryl-treated rabbits. The five ninhydrin-positive spots cochromatographed with metabolites B, E, F, and G, and carbaryl. Only two spots appeared with 1-naphthol administration that were not also present with corn oil administration; one of these spots corresponded to 1-naphthol, while the more polar unidentified metabolite did not correspond to any spot present from carbaryl treatment. Most of the metabolic reactions for carbaryl which yielded ether-extractable metabolites in the urine must, therefore, have taken place prior to in vivo hydrolysis of carbaryl to 1-naphthol.

Butanol extracts contained many materials only partially resolved by TLC. Four or five spots were obtained from urine of carbaryl-treated rabbits which were not found with the other types of treatment; these materials were different from any of the carbaryl metabolites recovered by ether extraction. 1-Naphthol treatment yielded two spots in the butanol extract not evident for the corn oil–treated or untreated rabbits. The identity of the compounds in the butanol extract following carbaryl or 1-naphthol treatment is unknown; however, they are assumed to be conjugates.

Chemical Nature of Carbaryl Metabolites. Metabolites of carbaryl or carbaryl-naphthyl-1-C^{14} obtained from the rat liver homogenate system or from rabbit urine were studied from a structural viewpoint. Compound H, as recovered finally by recrystallization from ether-hexane mixture, had an infrared spectrum and melting point (141° C.) identical with those of carbaryl. Metabolite I, as recovered finally by recrystallization from benzene-hexane mixture, yielded an infrared spectrum and melting point (96° C.) identical with those of 1-naphthol. Both of these two materials cochromatographed on two-dimensional TLC with the respective known compounds. Metabolite A was the arbitrary designation for material remaining at the origin of the TLC plates and the nature of this material was not investigated. Pure metabolite B was isolated. Metabolites C and G could not be isolated in amounts necessary for obtaining infrared spectra, and metabolites D, E, and F contained fluorescent impurities. These metabolites were used for bioassays and degradation studies. The fluorescent contaminant of each metabolite did not respond to the chromogenic agents or to the cholinesterase inhibition test.

Metabolite B in pure form consists of white needles (m.p. 159° C.), after recrystallization from acetone-hexane mixture. An infrared spectrum for this metabolite indicates that it is a *N*-methylcarbamate with additional hydroxyl functions and with loss of aromaticity of the naphthalene ring (see Figure 2). The band at 3.2-micron wavelength indicates a strongly hydrogen-bonded hydroxyl group and the band at 9.45-micron wavelength indicates a carbon-hydroxyl group configuration. Other bands of the spectrum are consistent with the proposed structure of a dihydrodihydroxycarbamate but, by themselves, are not definitive for establishing such a structure. This metabolite is degraded in alkali followed by acid treatment to a naphthalenediol by the following procedure: Digest

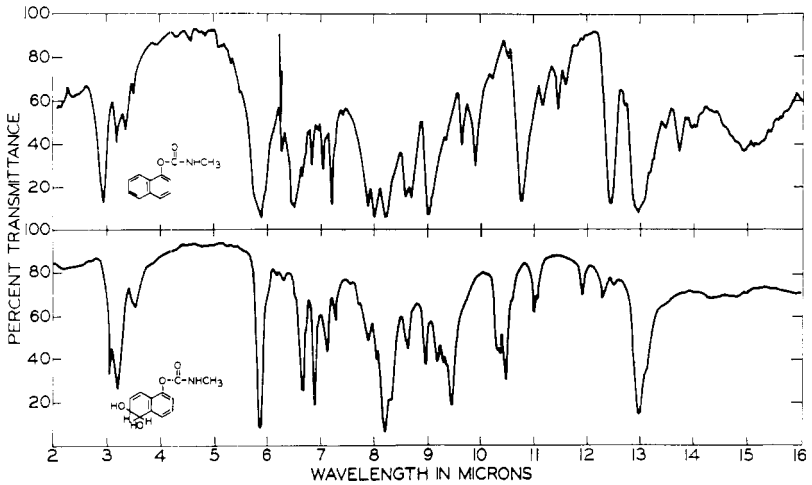

Figure 2. Infrared spectra for carbaryl and metabolite B recovered from rat liver enzymatic system and urine of carbaryl-treated rabbits

0.5 mg. at 25° C. for 4 hours in 1.0 ml. of 1*N* potassium hydroxide, extract with ether, and subject the ether extract to subsequent acid degradation by adding 1.0 ml. of 1 to 1 ethanol–concentrated hydrochloric acid mixture and allow it to react for 1 hour at 25° C.

The hydrolysis product cochromatographed with 1,5-naphthalenediol but not with several related compounds [including other naphthalenediols (1,3-; 1,4-; 1,6-; 1,7-; 2,3-; and 2,6-), 1,4-naphthoquinone, and 5-hydroxy-1,4-naphthoquinone]. The R_f values for these related compounds in 9 to 1 ether-hexane mixture are 0.61, 0.75, 0.69, 0.66, 0.64, 0.63, 0.77, and 0.75, respectively. In 2 to 1 chloroform-acetonitrile mixture, the R_f values are 0.58, 0.77, 0.66, 0.60, 0.53, 0.60, 0.78, and 0.75, respectively. The R_f of 1,5-naphthalenediol is 0.70 in the ether-hexane system and 0.69 in the chloroform-acetonitrile system. After similar treatment, metabolite B from carbaryl-naphthyl-1-C^{14} also yields 1,5-naphthalenediol-1-C^{14}, based on cochromatography. After saponification of metabolite B but prior to the acid treatment, none of these compounds, including 1,5-naphthalenediol, cochromatograph with the product. However, this product cochromatographs with metabolite D after it has been subjected to the same alkaline treatment; this is based on a light blue fluorescence under ultraviolet light and a blue color with Gibbs' reagent. The same material is formed from both metabolites B and D following saponification and treatment with acid, based on two-dimensional TLC. Both products also cochromatograph with 1,5-naphthalenediol and exhibit identical properties to this known compound in respect to fluorescence in long-wave ultraviolet light (after spraying with 1*N* methanolic hydrochloric acid and overspraying with 15% potassium hydroxide), and in yielding a light purple color when sprayed with Gibbs' reagent. It appears probable that metabolite D and the initial saponification product of metabolite B are both 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene, because this compound should decompose in acid to yield 1,5-naphthalenediol (*21*, *22*). Metabolite B is, therefore, 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate.

Metabolite C, which lacks the carbamate side chain (*7*), did not cochromatograph with any of the naphthalenediols previously mentioned, nor did the product derived when 0.5 mg. of C is treated with 1 ml. of 1 to 1 ethanol–concentrated hydrochloric acid mixture for 1 hour at 25° C. No other structural information is available on this metabolite.

Further studies on the nature of metabolites E, F, and G support the proposed structures of 1-naphthyl *N*-hydroxymethylcarbamate, 4-hydroxy-1-naphthyl methylcarbamate, and 5-hydroxy-1-naphthyl methylcarbamate, respectively (*7*). When 0.5 mg. of metabolite E is held in 1 ml. of 0.1*N* hydrochloric acid in methanol for 18 hours at 25° C., the product (following purification by TLC) cochromatographs on two-dimensional TLC with authentic 1-naphthyl carbamate. Both compounds weakly fluoresce light blue in long-wave ultraviolet light; this light blue fluorescence is greatly intensified by the potassium hydroxide spray, and subsequent spraying with Gibbs' reagent gives a dark blue color. Metabolite F yields 1,4-naphthalenediol and metabolite G yields 1,5-naphthalenediol when 0.5 mg. of each reacts with 1 ml. of 1*N* potassium hydroxide for 4 hours at 25° C., as ascertained by two-dimensional TLC. The product from F is also identical to 1,4-naphthalenediol in yielding a strong light blue fluorescence after potassium hydroxide treatment and a dark blue color when subsequently sprayed with Gibbs' reagent. Under the same conditions, the product from G is identical to 1,5-naphthalenediol in producing the strong dark blue fluorescence after alkali and the purple spots with Gibbs' reagent. Metabolite F labeled with C^{14} also cochromatographs with 1,4-naphthalenediol after similar saponification.

Biological Activity of Carbaryl Metabolites. Limited bioassays were made on carbaryl and its ether-extractable metabolites as recovered from the rat liver microsome plus soluble system and from rabbit urine. The susceptibility of houseflies to carbaryl, in terms of 24-hour LD_{50} in the presence of simultaneously applied piperonyl butoxide, was 0.05 μg. per female fly. No mortality occurred in 24 hours with flies treated with piperonyl butoxide and metabolite B, C, D, E, F, G, or I at 10 μg. per fly, nor with piperonyl butoxide and metabolite B, D, F, or I at 25 μg. per fly. The sensitivity limit for detection of metabolites by cholinesterase inhibition, as determined by direct assay on aluminum oxide plates (with human plasma as the enzyme source), is as follows: B = 0.3, C = 1.0, D = 0.5, E = 0.3, F = 0.1, G = 0.1, and I = 0.5 μg. carbaryl equivalents; that for carbaryl is 0.02 μg.

Metabolism of Carbaryl by Liver Microsomes. Table II shows the distribution of radioactivity found when carbaryl-naphthyl-1-C^{14} is incubated with $NADPH_2$ and the liver microsome

Table II. Carbaryl-naphthyl-1-C^{14} Metabolism by Liver Microsomes from Mice, Rabbits, and Rats in Presence of $NADPH_2$

Substance Determined	Total Radiocarbon, %, Using Liver Microsomes from Mice	Rabbits	Rats
Ether extract			
Carbaryl	32.1	19.4	46.9
Hydroxylated metabolites			
1-Naphthyl *N*-hydroxymethylcarbamate	11.9	6.3	11.7
4-Hydroxy-1-naphthyl methylcarbamate	6.7	8.1	6.1
5-Hydroxy-1-naphthyl methylcarbamate	2.4	1.7	1.3
5,6-Dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate	5.3	9.1	3.8
1-Hydroxy-5,6-dihydro-5,6-dihydroxy-naphthalene	1.9	2.7	1.5
1-Naphthol	7.2	6.3	5.8
Unidentified metabolites			
Metabolite A	4.0	4.5	3.5
Metabolite C	0.6	2.0	0.8
Aqueous fraction	28.5	39.6	19.4

fraction from mice, rabbits, and rats. In Table III, A, the activity of whole homogenates is compared with that of the microsome and soluble fractions, both individually and combined, without added cofactor and when fortified with $NADH_2$ or $NADPH_2$. The relative activity of the oxidized and reduced form of the cofactor in the presence of the microsome and the microsome plus soluble fractions is shown in Table III, B; the effect of $NADPH_2$ level on metabolism of carbaryl by these fractions is shown in Table III, C. The action of other cofactors, mostly NAD analogs, in the presence of the microsome plus soluble fraction is given in Table III, D. Table IV shows the effect

Table III. Carbaryl-naphthyl-1-C^{14} Metabolism by Rat Liver Fractions with Varying Cofactors

Metabolism Components	% Radiocarbon, in Ether Extract, Present as						Radiocarbon in Aqueous Phase, %
		Hydroxylated Carbamate Metabolites					
	Carbaryl	N-CH_2OH	4-OH	diH, diOH	Naphthol	Other metabolites	
	A. Fraction and Reduced Cofactor (2.0 μmoles) Varied[a]						
Whole homogenate							
No cofactor	78.7	2.0	1.3	0.4	8.0	1.5	8.2
$NADH_2$	27.0	5.4	4.7	2.4	6.9	1.8	52.3
$NADPH_2$	17.8	3.8	5.0	2.7	8.1	3.1	59.6
Microsome plus soluble							
No cofactor	55.0	5.7	3.8	0.5	8.1	1.3	25.6
$NADH_2$	16.2	3.2	4.1	0.6	6.4	1.4	68.2
$NADPH_2$	3.6	1.0	3.6	0.6	3.0	2.2	86.1
Soluble							
No cofactor	79.1	0.5	0.6	0.2	10.8	0.6	8.1
$NADH_2$	77.7	0.9	1.8	0.2	8.2	0.7	10.3
$NADPH_2$	73.9	0.3	1.9	0.2	14.6	0.6	8.7
Microsome							
No cofactor	75.8	2.3	4.6	0.2	6.1	1.0	10.0
$NADH_2$	41.6	9.0	9.6	2.6	12.5	2.9	22.7
$NADPH_2$	12.5	7.1	4.8	4.0	9.0	6.3	57.5
	B. Fraction and Oxidized and Reduced Cofactors (2.0 μmoles) Varied[b]						
Microsome							
NAD	88.5	0.5	0.2	0.2	7.6	0.5	2.7
$NADH_2$	67.3	4.3	3.5	1.0	10.6	1.3	12.3
NADP	90.2	0.1	0.1	0.1	7.6	0.2	1.9
$NADPH_2$	57.3	4.9	3.8	1.8	8.6	1.6	22.3
Microsome plus soluble							
NAD	52.6	4.4	4.5	1.6	6.7	2.5	27.8
$NADH_2$	8.6	3.4	1.8	1.4	6.9	3.1	74.9
NADP	37.8	5.9	6.7	0.6	6.5	3.3	39.2
$NADPH_2$	4.3	1.9	1.9	0.7	9.4	1.4	80.6
NAD plus NADP (1.0 μmole each)	21.4	4.6	4.5	0.7	6.6	5.3	56.9
	C. Fraction and Level (μmoles) of Reduced Cofactors Varied[a]						
Microsome							
$NADPH_2$, 0.25	81.1	2.0	2.4	0.9	9.3	1.3	3.0
$NADPH_2$, 0.50	78.0	2.4	3.0	1.2	8.6	1.5	5.4
$NADPH_2$, 1.0	67.2	4.9	5.1	2.0	9.2	2.4	9.4
$NADPH_2$, 2.0	53.0	7.8	7.4	3.4	10.0	3.3	15.2
$NADPH_2$, 4.0	43.4	11.1	9.1	3.7	8.6	4.1	20.0
$NADH_2$ plus $NADPH_2$ (0.50 each)	77.4	2.9	2.9	1.2	8.4	1.1	5.7
$NADH_2$ plus $NADPH_2$ (1.0 each)	68.5	5.7	4.9	2.0	7.7	2.0	9.3
Microsome plus soluble							
$NADPH_2$, 0.25	8.0	7.6	4.0	0.9	7.0	2.6	69.9
$NADPH_2$, 0.50	5.0	3.2	3.0	0.8	3.7	2.2	82.0
$NADPH_2$, 1.0	3.2	3.6	3.3	0.8	5.8	2.4	81.1
$NADPH_2$, 2.0	1.9	3.6	4.6	1.3	7.1	3.9	77.6
$NADPH_2$, 4.0	2.0	2.2	2.3	0.8	5.6	2.1	84.9
$NADH_2$ plus $NADPH_2$ (0.50 each)	11.6	4.6	7.3	0.9	8.2	2.7	64.7
$NADH_2$ plus $NADPH_2$ (1.0 each)	12.9	4.7	8.7	1.2	11.2	3.0	58.3
	D. Cofactors (2.0 μmoles) Varied with Microsome Plus Soluble Fraction[a]						
NAD	44.3	4.0	7.6	1.3	12.2	1.5	29.2
Deamino-NAD	16.8	2.9	8.0	2.5	12.4	2.5	55.5
3-Acetylpyridine-NAD	29.8	2.1	7.9	2.1	14.1	2.8	41.2
3-Acetylpyridine-deamino-NAD	20.5	1.7	7.3	2.4	13.9	2.4	51.7
3-Pyridinealdehyde-NAD	25.1	10.9	7.2	1.6	10.5	2.9	41.7
Thionicotinamide-NAD	25.8	6.6	8.2	1.6	13.6	1.9	42.3
Adenosine-5-mononicotinate	80.2	0.7	1.1	0.1	10.0	0.9	7.1
Nicotinamide mononucleotide	69.0	3.7	3.3	0.5	8.9	1.1	13.5
1-Methyl nicotinamide iodide	66.8	4.6	3.2	0.8	11.3	1.0	12.4
$NADPH_2$	1.0	4.7	2.0	1.3	3.7	4.1	83.2

[a] Average result from two experiments.
[b] Average result from three experiments.

Table IV. Carbaryl-naphthyl-1-C^{14} Metabolism by Rat Liver Microsome plus Soluble Fraction in Presence of NADP and Various Inhibitors[a]

Inhibitor and Molar Concentration	% Radiocarbon, in Ether Extract, Present as						Radiocarbon in Aqueous Phase, %
		Hydroxylated Carbamate Metabolites					
	Carbaryl	N-CH_2OH	4-OH	diH, diOH	Naphthol	Other metabolites	
None	9.2	3.0	5.7	0.8	8.6	2.4	70.3
Piperonyl butoxide							
1×10^{-5}	9.6	3.4	6.3	0.7	9.4	2.6	68.1
1×10^{-4}	30.8	5.5	8.0	0.9	9.9	2.2	42.7
1×10^{-3}	68.1	2.3	6.7	0.5	9.4	1.5	11.5
SKF-525A							
1×10^{-5}	20.4	4.4	6.3	0.5	8.8	1.6	57.9
1×10^{-4}	35.1	7.2	5.9	0.5	15.4	1.5	34.5
1×10^{-3}	51.1	3.3	4.0	0.4	17.3	1.6	22.4
MGK 264							
1×10^{-5}	15.6	10.1	7.4	0.8	9.7	5.6	51.0
1×10^{-4}	25.2	4.6	6.7	0.6	7.4	2.0	53.6
1×10^{-3}	50.7	2.6	3.9	0.5	5.8	1.2	36.7
Lilly 18947							
1×10^{-5}	11.8	3.1	7.0	0.9	9.8	2.6	64.8
1×10^{-4}	20.7	4.0	7.6	0.8	10.7	2.0	54.2
1×10^{-3}	63.5	2.1	5.1	0.5	7.6	1.3	19.9
2-(2,4-Dichloro-1-naphthoxy)ethyldiethylamine							
1×10^{-5}	17.0	4.2	4.4	0.7	9.1	2.3	62.3
1×10^{-4}	16.8	5.4	5.6	0.8	8.5	2.4	60.6
1×10^{-3}	39.9	8.1	9.7	0.7	15.4	2.0	24.3

[a] Average result from two experiments.

of inhibitors on carbaryl metabolism by the microsome plus soluble fraction, in the presence of NADP. Supporting data are available for other studies discussed below without the benefit of a tabulation of results (*17*).

Incubation of carbaryl with liver microsomes from mice, rabbits, and rats, in the presence of $NADPH_2$, produces the same variety of metabolites (Table II). (The data tabulated in Table II are average results from duplicate experiments using microsomes from two young and two old animals of each sex; thus, they are the average from eight experiments in all.) Carbaryl metabolism is the greatest with rabbit, next with mouse, and least with rat microsomes. Microsomes from adult and young male rabbits give more metabolism than those from comparable female rabbits; conversely, microsomes from adult and young female mice give more metabolism than those from comparable male mice. No such sex difference is evident with rat microsomes (*17*). Carbaryl metabolism was also examined with selected housefly and American cockroach [*Periplaneta americana* (L.)] tissues, using the microsome fraction as prepared from 10% homogenates by using the same centrifugation series employed to separate liver microsomes. Insect microsomal fractions, fortified with $NADPH_2$, appear to form the same metabolites as liver microsomes, based on TLC, but in such small quantities that correlations cannot be established with certainty (*17*).

Of particular significance is the finding of the same ether-extractable carbaryl metabolites with microsomes of each species, based on TLC (Table II). The same is true of other fractions obtained from rat liver (Table III). Apparently, in vitro detoxication involves: hydroxylation of the *N*-methyl group to yield 1-naphthyl *N*-hydroxymethylcarbamate (N-CH_2OH); hydroxylation of the 4- and 5-positions of the naphthyl ring to yield 4-hydroxy-1-naphthyl methylcarbamate (4-OH) and 5-hydroxy-1-naphthyl methylcarbamate (usually tabulated with other metabolites); epoxidation of the 5,6-position of the naphthyl ring with subsequent decomposition of the epoxide to yield 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate (diH,diOH); hydrolysis of the latter compound to yield 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene (usually tabulated with other metabolites); hydrolysis of carbaryl or the *N*-hydroxymethylcarbamate to yield 1-naphthol; and formation of one unidentified carbamate (metabolite A) and one unidentified hydrolysis product (metabolite C) (usually tabulated with other metabolites). These various types of attack on the carbaryl molecule can be effected by the same liver microsome–$NADPH_2$ system. In this consideration, it is important to know the optimum conditions for carbaryl metabolism by liver enzymes and to determine not only the loss of carbaryl but also the type of metabolite formed.

As shown in Table III, A, optimum activity for carbaryl destruction results in the presence of microsome plus soluble fractions fortified with $NADPH_2$. The soluble fraction alone [and the debris fraction alone (*17*)], with or without cofactor fortification, are essentially inactive in catalyzing reactions other than minimal hydrolysis to 1-naphthol. Dialysis of the liver soluble fraction further decreases its activity for carbaryl metabolism, both alone and when fortified with $NADPH_2$; boiling the liver soluble prior to assay destroys all of its activity, with and without $NADPH_2$. The whole homogenate is much more active in carbaryl destruction than comparable reconstituted homogenates lacking either the microsome or soluble fractions without fortification and with either added $NADH_2$ or $NADPH_2$ [Table III, A, and (*17*)]. The activity for formation of hydroxylated metabolites appears to reside largely in the microsome fraction. The activity of the microsome fraction is enhanced by addition of the soluble fraction, particularly in respect to increasing the proportion of radiocarbon recovered in the aqueous phase (Table III, A); from other experiments, it is known that the quantity of labeled material in the aqueous fraction varies directly with the quantity of solubles in the incubation mixture from 0.25 to 1.0 ml. (*17*). The effect of the soluble fraction when added to the microsome fraction in increasing the proportion of the radiocarbon in the aqueous phase is diminished on dialysis of the soluble fraction and, to a greater—but not complete—extent, by boiling the soluble fraction.

Comparison of the oxidized and reduced forms of NAD and NADP, and the effect of certain NAD analogs, on carbaryl metabolism by the microsome or microsome plus soluble fractions indicates that $NADPH_2$ is the most effective cofactor (Table III, B, C, and D). The reduced cofactors ($NADH_2$ or $NADPH_2$) are better than the corresponding oxidized cofactors (NAD or NADP). This is clear with the microsome plus soluble fraction, but it is particularly evident with the microsome fraction alone in cases where appreciable carbaryl metabolism occurs only with the

reduced cofactor (Table III, B). The extent of carbaryl metabolism by both the microsome and microsome plus soluble fractions is enhanced by increasing levels of $NADPH_2$ (Table III, C). Mixtures of cofactors differ in their effect in some cases as compared with individual cofactors—for instance, NAD and NADP mixtures are more effective with the microsome plus soluble fraction than either cofactor alone, at equivalent total levels (Table III, B); $NADH_2$ and $NADPH_2$ mixtures are less effective with the same type of enzyme preparation than either cofactor alone; however, with the microsome fraction alone, the activity of $NADH_2$ and $NADPH_2$ mixtures is approximately equivalent to the $NADPH_2$ amount present (Table III, C). From tests made to study the cofactor specificity of the microsome plus soluble fraction in carbaryl metabolism, it is evident that each NAD analog gives greater carbaryl metabolism than NAD, and the two deamino compounds give more metabolism than the corresponding amino compounds (Table III, D). Some activity, although small, is also found with adenosine-5-mononicotinate and 1-methyl nicotinamide iodide (Table III, D).

Other variables studied include the pH optimum, divalent cation effect, and nicotinamide requirement of the system (*17*); the standard incubation mixture, described under Methods and Materials, was varied by altering the pH of the phosphate or by adding the other constituents. Carbaryl metabolism occurring in the presence but not in the absence of $NADPH_2$ is optimal with a phosphate buffer at pH 7.3 for both the microsome and microsome plus soluble fractions. This same pH optimum (7.3) is evident when the extent of metabolism is based on residual carbaryl or on radiocarbon in the aqueous fraction with both types of enzyme preparations. Recovery of each individual metabolite is not always greatest at pH 7.3 because, with microsomes, the proportion of the carbaryl recovered as 1-naphthyl *N*-hydroxymethylcarbamate relative to that recovered as 4-hydroxy-1-naphthyl methylcarbamate shifts with decreasing hydrogen ion concentration in favor of the latter compound. Variations in stability of the several carbamate metabolites in the alkaline region may contribute to such differential metabolite recovery. Naphthol recovery from the standard incubation mixture is not altered by $NADPH_2$ or the type of enzyme preparation and is as follows, at various pH values: 4% at 6.0, 5% at 6.3, 8% at 7.0, 10% at 7.3, 16% at 8.0, and 18% at 8.3. In regard to the effect of selected divalent cations on carbaryl metabolism by liver microsomes fortified with $NADPH_2$ (in the presence and absence of nicotinamide), neither the cations at 1×10^{-3} *M* nor the nicotinamide at 8×10^{-3} *M* greatly affect the extent of carbaryl metabolism. Metabolism appears to be increased slightly by barium, cobalt, and calcium, but is inhibited by magnesium and manganese. (Barium was used routinely in incubation mixtures because the amount of ether-extractable metabolites formed by the microsome fraction was greatest with this cation and because the extent of carbaryl metabolism was primarily based upon TLC resolution and analysis of the metabolites in the ether extract of incubation mixtures.) Nicotinamide does not alter the $NADPH_2$-catalyzed carbaryl metabolism by microsomes, but it was routinely added in all studies because it might minimize NAD and NADP destruction from the action of NAD nucleosidase.

The effect of selected inhibitors at 10^{-5}, 10^{-4}, and 10^{-3} *M* on metabolism of carbaryl by the microsome plus soluble fraction fortified with NADP and by the microsome fraction fortified with either $NADPH_2$ or $NADH_2$ is known [Table IV and (*17*)]. Inhibition varies directly with the concentration of each inhibitor and, usually, is slight at the 1×10^{-5} *M* levels but marked at the 1×10^{-3} *M* levels. Results with the microsome plus $NADH_2$ and microsome plus $NADPH_2$ studies are similar in extent of inhibition by the synergists, and the SKF-525A and MGK 264 are more active than the other compounds. Little if any inhibitor specificity is evident for the position of hydroxylative attack blocked by the different synergists.

Conjugation of Carbaryl Metabolites by Liver Enzymes. Table V gives a picture of the ether-extractable metabolites of carbaryl, recovered after formation by the liver microsome plus $NADPH_2$ system, and subsequent reincubation with various conjugation systems. A selective reduction in amount of any metabolite, in the presence of a particular conjugation system, indicates the formation, from that metabolite, of the type of conjugate appropriate for the system. (Systems examined included those for glutathione conjugation, glucuronide conjugation, and sulfate conjugation.) The presence of the different cofactors in the various conjugation systems in the absence of liver fractions does not greatly alter the ratio of metabolites. In the absence of conjugation cofactors, the presence of the microsome or soluble fraction does not alter the metabolite ratio, and the effect of the microsome plus soluble fraction in the absence of conjugation cofactors is small. The glutathione conjugation system has little or no effect on the ratio of metabolites in the presence of any of the liver fractions.

Table V. Conjugation of Ether-extractable Metabolites of Carbaryl-naphthyl-1-C^{14} by Rat Liver Fractions with Selected Cofactors[a]

Liver Fraction and Conjugation System	% Radiocarbon, in Ether Extract, Present as						Radiocarbon in Aqueous Phase, %
		Hydroxylated Carbamate Metabolites					
	Carbaryl	N-CH_2OH	4-OH	diH, diOH	Naphthol	Other metabolites	
No liver fraction							
No conjugation system	22.3	8.7	9.4	2.3	34.6	9.6	13.1
Glutathione conjugation system	24.3	10.6	13.0	2.3	30.9	8.4	10.6
Glucuronide conjugation system	23.9	10.8	11.1	2.6	31.9	9.8	9.9
Sulfate conjugation system	25.2	13.1	13.1	2.3	29.2	10.3	6.8
Microsome							
No conjugation system	18.1	9.4	13.4	2.3	34.2	8.4	14.4
Glutathione conjugation system	18.3	8.2	12.3	1.7	30.8	7.0	21.8
Glucuronide conjugation system	16.1	7.7	3.9	1.4	9.6	4.3	57.1
Sulfate conjugation system	20.6	12.6	13.4	1.8	32.3	8.0	11.5
Soluble							
No conjugation system	23.1	11.2	12.7	1.8	29.9	6.9	14.4
Glutathione conjugation system	21.8	9.2	13.8	1.7	32.3	6.9	14.3
Glucuronide conjugation system	19.6	7.2	4.5	1.3	5.8	4.3	57.5
Sulfate conjugation system	25.7	12.5	8.8	1.9	11.1	5.5	36.7
Microsome plus soluble							
No conjugation system	18.0	7.8	12.4	1.7	27.0	6.1	27.0
Glutathione conjugation system	21.1	9.9	13.6	1.6	28.8	5.7	19.2
Glucuronide conjugation system	21.2	9.3	6.3	1.6	11.1	4.7	45.9
Sulfate conjugation system	20.3	9.2	9.2	1.7	18.7	6.3	34.6

[a] Average result from two experiments.

The sulfate conjugation system has no effect in the presence of the microsome fraction alone but, with the soluble or microsome plus soluble fractions, it results in loss of naphthol and 4-hydroxy-1-naphthyl methylcarbamate. With the soluble and microsome plus soluble fractions, it also results in a reduction in amount of 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene and, possibly, metabolite C (*17*).

Glucuronide conjugation appears to be of greatest significance (or the conditions for conjugation are more optimal).

Minimal loss of 1-naphthyl *N*-hydroxymethylcarbamate and 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate results with the glucuronide conjugation system containing the microsome or soluble fraction. Marked loss occurs with all the liver fractions fortified with uridine diphosphoglucuronic acid in recovery of the following metabolites: 1-naphthol and 4-hydroxy-1-naphthyl methylcarbamate (Table V); among the other metabolites, loss occurs in recovery of 5-hydroxy-1-naphthyl methylcarbamate, 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene, and unidentified metabolites A and C (*17*). The conjugates are probably present in the aqueous phase following extraction with ether because the radiocarbon shifts into this phase when ether-extractable metabolites are altered by the conjugation systems. The addition of cofactor components for the conjugation systems to the standard carbaryl plus $NADPH_2$ microsomal incubation mixtures gave no evidence of metabolite conjugation (*17*).

Discussion

The chemical nature of the ether-extractable carbaryl metabolites as proposed by Dorough and Casida (*7*) is largely confirmed by the results of the present study, in which the types of characterization evidence are greatly expanded. The structures for metabolites B and D differ from those speculated for them, which were based almost entirely on analogy with the known naphthalene metabolic pathway. Metabolite B appears to be 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate and metabolite D the hydrolysis product of B—i.e., 1-hydroxy-5,6-dihydro-5,6-dihydroxynaphthalene. The principal epoxide intermediate would therefore be the 5,6-epoxide of 1-naphthyl methylcarbamate, rather than the 3,4-epoxide depicted by Dorough and Casida (*7*). Since 4-hydroxy-1-naphthyl methylcarbamate is also present as a carbaryl metabolite, it is possible that the 3,4-epoxide of 1-naphthyl methylcarbamate is also formed but fails to decompose to yield significant amounts of 3,4-dihydro-3,4-dihydroxy-1-naphthyl methylcarbamate.

Still remaining to be elucidated is the chemical nature of metabolite C, and of three or four additional ether-extractable, and four or five additional 1-butanol-extractable carbaryl metabolites that are present in the urine of carbaryl-treated rabbits, but not in the urine of 1-naphthol–treated rabbits. It is possible that the 1-butanol–extractable materials are conjugates of certain carbamate metabolites of carbaryl; however, none of these compounds are available in a pure form (for characterization). The relation between the metabolites in the aqueous fraction from the in vitro studies and the 1-butanol fraction from the urine studies is not known because 1-butanol was not used for extraction of the enzymatic reaction mixtures. Experiments using the "preformed metabolites" containing hydroxyl groupings indicate the possible formation of the following conjugates: glucuronides from each of the eight ether-extractable carbaryl metabolites; ethereal sulfates from 4-hydroxy-1-naphthyl methylcarbamate and the three hydrolysis products which are unesterified naphthol derivatives. In a recent report (*14*), the glucuronides and sulfates of 1-naphthol and 4-hydroxy-1-naphthyl methylcarbamate were identified in the urine of carbaryl-treated rats and guinea pigs. At least 15 carbaryl metabolites have been demonstrated by urinalysis in the present study, and enzymatic studies give evidence for the formation of at least 20 carbaryl metabolites.

The pathway of carbaryl metabolism may be general in mammals because enzymatic systems from livers of mice, rabbits, and rats give many of the same products which appear in the urine of rabbits [and, also, of goats (*7*)]. Only seven of these metabolites have been subjected to bioassay using houseflies and plasma cholinesterase. From the limited bioassay data available from the present and a previous study, it appears that the metabolites are of little toxicological importance in consideration of residue levels or hazards as compared with the more active precursor, carbaryl (*7*).

The nature of the liver microsome–$NADPH_2$ system that metabolizes carbaryl is similar to that attacking many drug types, in particular to those hydroxylating naphthalene and the *N*-methyl group of *p*-nitrophenyl dimethylcarbamate (*3*, *11*, *13*). Since $NADH_2$ as well as $NADPH_2$ is effective, it appears that the enzymatic reaction is not cofactor specific and the possibility exists, but does not necessarily follow from the results, that different enzymes are involved with the two cofactors. Several NAD analogs give greater carbaryl metabolism than NAD with the microsome plus soluble fraction. It is not known whether the NAD analogs are more efficient cofactors than NAD because of greater ease of reduction by the soluble fraction, or greater stability during incubation, or if the reduced products formed are more effective cofactors than $NADH_2$ for the hydroxylation system. Naphthol liberation during the enzymatic reactions appears to be independent of added pyridine nucleotide cofactor, indicating that it originates primarily from nonenzymatic hydrolysis of carbaryl, possibly catalyzed by the phosphate buffer (*5*).

The soluble fraction enhances the metabolism of carbaryl by the microsome plus reduced pyridine nucleotide cofactor system. This effect of the soluble fraction is diminished by dialysis and largely destroyed by boiling. A part of the action of the soluble fraction probably results from providing the cofactors or enzymes for conjugation of the hydroxylated compounds formed by the microsomes. The synergists or potential metabolic inhibitors may reduce carbaryl detoxication by serving as competitive substrates for the liver microsome–$NADPH_2$ system. Naphthalene metabolism, by housefly microsomes and $NADPH_2$, is also inhibited by levels of piperonyl butoxide and SKF-525A similar to those found, in the present study, to inhibit carbaryl metabolism by rat liver microsomes and $NADPH_2$ (*20*). Hydroxylation of one *N*-alkyl group of *p*-nitrophenyl dimethyl- and diethylcarbamates by liver microsomes plus $NADPH_2$ is inhibited by piperonyl butoxide, MGK 264 and SKF-525A (*13*). Each of these synergists is known to be effective in increasing the insecticidal activity of carbaryl or other insecticides, possibly by reducing the detoxication rate in the insect (*10*, *12*, *18*, *19*, *20*, *24*). On the basis of the present studies, the inhibition of carbaryl detoxication appears to be general rather than selective for one of the sites of hydroxylation on the molecule.

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METABOLISM IN PLANTS

Fate of C^{14}-Carbonyl-Labeled Aryl Methylcarbamate Insecticide Chemicals in and on Bean Plants

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Studies with eight C^{14}-carbonyl-labeled aryl methylcarbamates demonstrate that such insecticide chemicals are degraded with the carbamate moiety intact when applied to glass or silica gel surfaces or leaves of growing bean plants, or injected into the stems of such plants. Methylcarbamates-carbonyl-C^{14} of the following phenols were examined: 1-naphthol (carbaryl), 2-isopropoxyphenol (Baygon), 3-isopropylphenol (UC 10854), 3,5-diisopropylphenol (HRS-1422), 2-chloro-4,5-xylenol (Banol), 4-methylthio-3,5-xylenol (Mesurol), 4-dimethylamino-3,5-xylenol (Zectran), and 4-dimethylamino-3-cresol (Matacil). Rates of loss vary considerably with the nature of the surface (inert or plant), the light, and the compound. Oxidative changes occur on and/or in the plant: Mesurol degrades to sulfoxide and sulfone analogs; Zectran and Matacil form several methylcarbamate derivatives, including the 4-methylamino, 4-amino, 4-methylformamido, and 4-formamido analogs. Little or no formation of organoextractable degradation products, containing an intact methylcarbamate moiety, occurs with the other compounds. The fate of the radiocarbon, 6 days after injection into bean plants, varies considerably: With Baygon and UC 10854 the majority of the radiocarbon is in the water phase; with Zectran and Matacil it appears in the unextractable portion; with Mesurol, it shows up as loss, possibly as the result of expiration as $C^{14}O_2$. Loss from the plant surface is not directly related to the volatility of the compounds, nor is degradation in the plant related to the rate of nonenzymatic hydrolysis. It appears that the relative stability of the methylcarbamate grouping to photooxidation and metabolism, in certain cases, allows the formation of degradation products involving only alteration of the ring or a ring substituent.

CERTAIN aryl methylcarbamates are commercial or experimental insecticide chemicals for the control of insects on a variety of agricultural crops, including food crops. To evaluate the potential hazard from their use, it is necessary to know their fate in and on plants, especially if they have a systemic action or if their use leaves a persistent residue, because the methods used to determine residues must measure products formed by metabolism or degradation in or on the plant, when these are also toxic, as well as the original chemical used.

Carbaryl (1-naphthyl methylcarbamate), in the formulated state, slowly degrades when irradiated by ultraviolet light and sunlight to unidentified products as analyzed by ultraviolet absorption at 280 mμ (*19*). Under exposure to ultraviolet light (2537 A.) on paper chromatograms, isolan (1-isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate) and pyrolan (1-phenyl-3-methyl-5-pyrazolyl dimethylcarbamate) give rise to several unidentified products as detected by chromogenic agents (*17*). Recently, Crosby, Leitis, and Winterlin stated that methylcarbamates produce a variety of unidentified cholinesterease-inhibiting derivatives when exposed as ethanol or hexane solutions to ultraviolet light at 254 mμ or to sunlight (*2*). Matacil (4-dimethylamino-3-cresyl methylcarbamate) and Zectran (4-dimethylamino-3,5-xylyl methylcarbamate) extensively degrade to five or more inhibitory products. Mesurol (4-methylthio-3,5-xylyl methylcarbamate) gives at least two in-