Metabolites of Carbaryl (1-Naphthyl Methylcarbamate) in Chicken Urine

G. D. Paulson, R. G. Zaylskie, M. V. Zehr, C. E. Portnoy, and V. J. Feil

Hens, surgically modified to facilitate collection of urine, were given a single oral dose of carbaryl-1-naphthyl 1-^{14}C, carbaryl-carbonyl-^{14}C, or carbaryl-methyl-^{14}C (100 mg./kg. body weight), and urine was collected for 6 hours. Urinary metabolites of carbaryl were separated and purified by solvent extraction, thin-layer chromatography, and column chromatography. Acetyl derivatives of the metabolites were purified by gas chromatography. Metabolites and derivatives were characterized by infrared, ultraviolet, and mass spectrometry. Confirmation of structure was made by *in vitro* biosynthesis. Urinary metabolites which were identified were 1-naphthol, 1-naphthyl glucuronide, and the sulfate esters of 1-naphthol, 4-hydroxycarbaryl, and 5-hydroxycarbaryl. Characterization of the hydrolysis products and acetyl derivatives of the other metabolites indicated that two were conjugates of 1,5-naphthalenediol, one was conjugated 4-hydroxycarbaryl, one was conjugated 5-hydroxycarbaryl, one was conjugated 5,6-dihydroxycarbaryl, one was conjugated 1,5,6-trihydroxynaphthalene, and two were conjugates of carbaryl.

Carbaryl (1-naphthyl methylcarbamate) is used extensively to control ectoparasites on poultry (Harrison, 1961; Agriculture Handbook, 1968). The toxicity of carbaryl in chickens has been studied (Sherman and Ross, 1961). Tissues and eggs have been analyzed for carbaryl and naphthol after chickens were treated with carbaryl by dusting (Johnson *et al.*, 1963), and by oral dosing (Furman and Pieper, 1962; McCay and Arthur, 1962; and Nir *et al.*, 1966). When mature chickens were given a single oral dose of carbaryl-naphthyl-1-^{14}C, most of the activity was excreted in the urine, although 1.4% of the radioactivity remained in the birds 48 hours after the dose was given (Paulson and Feil, 1969). Although the metabolites of carbaryl in mammals have been studied (Leeling and Casida, 1966; Knaak *et al.*, 1965; Dorough and Casida, 1964), information concerning the metabolites of carbaryl in avian species is not available. The purpose of the study reported here was to isolate and identify the metabolites of carbaryl in chicken urine.

EXPERIMENTAL

Chemicals and Supplies. The sources of chemicals and supplies were as follows: analytical grade carbaryl, 4-hydroxycarbaryl, 5-hydroxycarbaryl, and 5-hydroxy-1,2-naphthoquinone, Union Carbide Corp., Chemicals and Plastics Division; 1,5-naphthalenediol, 1,4-naphthalenediol, and methanesulfonic acid, Eastman Organic Chemicals, Eastman Kodak Company; $H_2{}^{35}SO_4$, New England Nuclear Corp.; adenosine-5′-triphosphate, 1-naphthyl sulfate, 1-naphthyl-β-D-glucuronic acid, and Na_2HPO_4, Sigma Chemical Company; β-glucuronidase, aryl sulfatase (*Helix pomatia*), Calbiochem; silica gel G, Research Specialties Company; Gas Chrom Q, 80–100 mesh and OV-1, Applied Science Laboratories, Inc.; Sephadex G-10 and Sephadex LH-20, Pharmacia Fine Chemicals, Inc.; NCS digestion reagent and carbaryl-carbonyl-^{14}C, Amersham/Searle Corp.; methanol, 1-naphthol, $MgCl_2$, and K_2SO_4, Fisher Scientific Co.

Synthesis of Chemicals. Carbaryl-naphthyl-1-^{14}C and carbaryl-methyl-^{14}C were synthesized and purified as previously described (Krishna *et al.*, 1962; Paulson and Feil, 1969). The method of Sullivan *et al.* (1967) was used to synthesize the following compounds: *N*-acetylcarbaryl, 5-acetoxy *N*-acetylcarbaryl, 1,5-diacetoxynaphthalene, and 1,4-diacetoxynaphthalene. The method of Sullivan *et al.* (1967) was modified by the addition of an excess of Zn dust to the acetylating reagent for the synthesis of 1,5,6-triacetoxynaphthalene from 5-hydroxy-1,2-naphthoquinone.

Biosynthesis of ^{35}S-Labeled Metabolites. Mature Leghorn hens were sacrificed by decapitation; the livers were removed, cooled on ice, and homogenized in 0.1*M*, pH 6.5 phosphate buffer (1 part fresh liver, 6 parts buffer w./v.). The homogenate was centrifuged at 105,000 × G for 45 minutes with a Beckman Model L-2 centrifuge (type 50 rotor); the supernatant fraction was used immediately or stored at −10° C. until used. The reaction mixture contained the following: 5 ml. of 0.078*M* $MgCl_2$; 5 ml. of 0.048*M* K_2SO_4 (40 μCi. of $^{35}SO_4$); 5 ml. of 0.084*M* adenosine-5′-triphosphate (ATP); 10 ml. of 105,000 × G supernatant fraction; 0.2 ml. of polyethyleneglycol 400; 5 ml. of H_2O; 200 μg. of either naphthol, 4-hydroxycarbaryl, or 5-hydroxycarbaryl. The reaction mixture was incubated at 39° C. for 2 hours, and then the reaction was stopped by the addition of 30 ml. of methanol. The methanol and water were removed on a flash evaporator, and the residue which remained was washed with 10 ml. of H_2O. The water wash was extracted twice with an equal volume of benzene, and the benzene extract discarded. The aqueous fraction was then evaporated to dryness on a flash evaporator, and the residue which remained was extracted three times with 20 ml. of methanol. The methanol extracts were combined and evaporated to dryness on a flash evaporator at 40° C. The residue was dissolved in a small amount of water and applied to a 1- × 60-cm. Sephadex G-10 column poured in water; the ^{35}S-labeled compound was then eluted with water (flow rate, 0.2 to 0.3 ml./min.), and the effluent was collected in 2.5-ml. fractions. The radiolabeled fraction from the Sephadex G-10 column was evaporated to dryness, dissolved in a small amount of methanol, and applied to a 1- × 110-cm. Sephadex LH-20 column poured in methanol. The ^{35}S-labeled compound was eluted with methanol (flow rate, 0.2 to 0.3 ml./min.), and the effluent was collected in 1.2-ml. fractions. The fractions which contained the radiolabeled compound were concentrated to a small volume for characterization studies.

Treatment of Birds. Mature white Leghorn hens, surgically modified to facilitate separate collection of urine (Paulson, 1969), were given a single oral dose of carbaryl-naphthyl-1-^{14}C, carbaryl-carbonyl-^{14}C, or carbaryl-methyl-

Animal Husbandry and Entomology Research Divisions, U. S. Department of Agriculture, Metabolism and Radiation Research Laboratory, Agricultural Research Service, Fargo, N. D. 58102

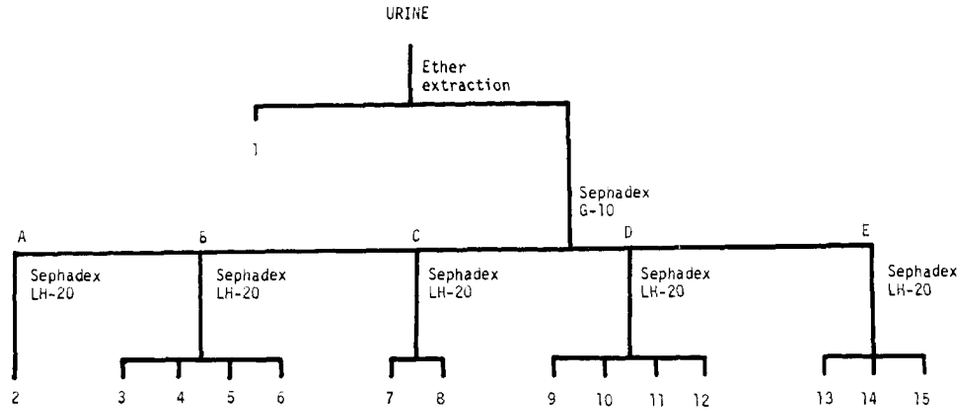


Figure 1. Separation and purification of carbaryl metabolites in chicken urine

^{14}C. The dose contained from 10 to 15 μCi. of carbon-14 and was made to supply 100 mg. of carbaryl per kg. of body weight by the addition of an appropriate amount of unlabeled carbaryl. Urine was collected for 6 hours after the dose was administered. The hens had free access to distilled water and to a commercial 16% protein laying mash for several weeks prior to and throughout the experimental period.

Purification of Urinary Metabolites. The urine was filtered, and the filtrate was extracted three times with an equal volume of diethyl ether (Figure 1). The ether phase was concentrated to a small volume with a flash evaporator and applied to a silica gel G thin-layer plate; the chromatogram was developed with hexane-acetone (4:1, v./v.), and the plate was scanned with a Packard Model 7200 radiochromatogram scanner to determine the location of the carbon-14. The radiolabeled region was scraped from the plate into a test tube, and the carbon-14-labeled compound was extracted from the silica gel with benzene. The extract was concentrated to a small volume on a flash evaporator at 40° C. for characterization studies.

After ether extraction, the aqueous phase was evaporated to dryness on a flash evaporator at 40° C. The residue which remained was repeatedly extracted with 10-ml. aliquots of methanol until at least 95% of the activity was removed, and the last extract contained less than 1% as much carbon-14 as the previous extract. The combined methanol extracts were evaporated to dryness on a flash evaporator at 40° C.; the residue was dissolved in a small amount of water and applied to a 2.5- × 110-cm. Sephadex G-10 column poured in water. The radiolabeled metabolites were eluted with water (flow rate, 0.5 ml./min.), and the effluent was monitored with a Model 2002 Packard liquid scintillation spectrometer equipped with a continuous flow cell, ratemeter, and strip chart recorder. Each of the five radiolabeled fractions which were eluted from the Sephadex G-10 column (*A*, *B*, *C*, *D*, and *E* in Figure 1) were then evaporated to dryness on a flash evaporator at 40° C. The residue from each fraction from the G-10 column was dissolved in a small amount of methanol and applied to a 1- × 110-cm. Sephadex LH-20 column poured in methanol. The radiolabeled metabolites were eluted from the LH-20 column with methanol (flow rate, 0.2 to 0.3 ml./min.), and the effluent was collected in 1.2-ml. fractions. Each fraction which contained a radiolabeled metabolite was concentrated to a small volume on a flash evaporator for characterization studies.

Enzyme Hydrolysis. A tube containing from 100 to 200 μg. of the metabolite, 0.1 ml. of enzyme solution (0.7 EU of β-glucuronidase and 0.3 EU of aryl sulfatase), and 0.5 ml. of 0.1*M* sodium acetate buffer (pH 4.5) was incubated at 39° C. for 30 minutes. The radiolabeled hydrolysis product was then extracted from the mixture with 0.5 ml. of benzene and characterized by infrared spectrometry.

Acetylation of Metabolites. The method of Paulson and Portnoy (1970) was used to replace polar conjugates on the urinary metabolites with an acetyl group. The conjugated metabolites (approximately 200 μg.) were mixed with 0.05 ml. of the acetylating reagent (40 parts acetic anhydride and 1 part methanesulfonic acid v./v.) at 0° C. The mixture was heated at 100° C. for 30 min. and then cooled to 0° C. A small amount of ice (equivalent to approximately 0.2 ml. of water) was added, and the mixture was extracted three times with an equal volume of benzene. The combined benzene extracts were concentrated to a small volume, and the acetylated compounds were purified using a Barber-Colman Series 5000 gas chromatograph equipped with a hydrogen flame detector and a radioactivity monitoring system (column, 8 ft., 5 mm. I.D.; support, Gas Chrom Q, 80–100 mesh; liquid phase, 2% OV-1; helium flow rate, 55 ml./min.; injection port temperature, 350° C.; detector temperature, 350° C.; temperature programmed from 120° to 300° C. at 5° C./min.). Capillary tubes were used for trapping the acetylated compounds from the gas chromatograph.

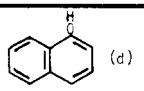
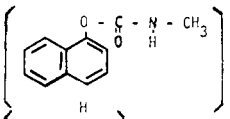
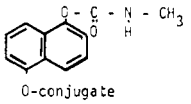
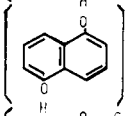
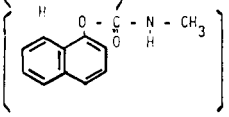
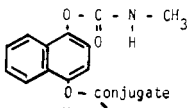
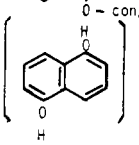
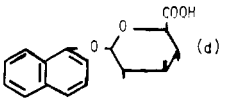
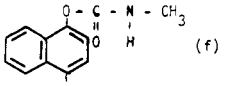
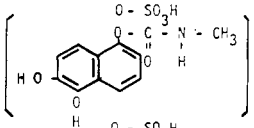
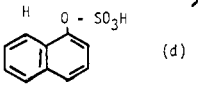
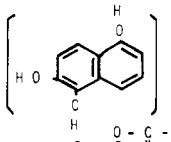
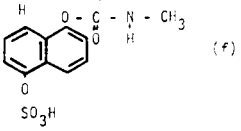
Instrumental Analysis. Infrared spectra were taken with a Model 337 Perkin-Elmer grating infrared spectrometer using the micro KBr technique (1.5-mm. disk with a 4× beam condenser). Ultraviolet spectra were taken with a Bausch & Lomb Spectronic 505 spectrometer. Mass spectra were taken with a Varian M-66 mass spectrometer equipped with a V-5500 interface control console. The mass spectra were recorded with either a Midwestern Model 801 oscillographic recorder or a Varian Statos Model 153 electrographic recorder.

RESULTS AND DISCUSSION

From 75 to 85% of the ^{14}C-activity administered was excreted in the urine during the 6-hour collection period; this observation was in agreement with previous studies on carbaryl metabolism in chickens (Paulson and Feil, 1969).

Only one radioactive compound was removed from the urine by ether extraction. This compound was purified by thin-layer chromatography and identified as 1-naphthol by comparing the infrared spectrum with that of an authentic sample. The amount of 1-naphthol in the urine varied from 0 to 15% of the ^{14}C-labeled metabolites; however, in most instances it accounted for less than 1% of the activity.

Table I. Characterization of Carbaryl Metabolites Isolated from Chicken Urine

Metabolite	Percent of Urinary 14-Carbon (range)	Carbonyl[a] Carbon Present	Methyl[b] Carbon Present	Acetylation Products[c]	Structure
1	0-15	no	no	naphthyl acetate	 (d)
2	1-2	?	?	NR[e]	?
3	5-9	yes	yes	N-acetyl carbaryl 5-acetoxy N-acetyl carbaryl	 conjugate  O-conjugate
4	1-6	no	no	1, 5-diacetoxy naphthalene	 conjugate
5	1-5	yes	yes	N-acetyl carbaryl	 conjugate
6	0-1	?	?	NR	?
7	2-7	yes	yes	NR - hydrolyzed to 4-OH carbaryl enzymatically	 O - conjugate
8	3-4	no	no	1,5-diacetoxy naphthalene	 conjugate
9	0-6	?	?	NR	?
10	6-16	no	no	NR	 (d)
11	7-12	yes	yes	4-acetoxy N-acetyl carbaryl 1,4-diacetoxy naphthalene	 (f)
12	3-8	yes	yes	1,5,6-triacetoxy naphthalene	 conjugate
13	41-46	no	no	naphthyl acetate	 (d)
14	2-3	no	no	1,5,6-triacetoxy naphthalene	 conjugate
15	0-5	yes	yes	not conducted	 (f)

[a] Verified by isolation of (or failure to isolate) a metabolite with a ^{14}C-label in the carbonyl position which gave an infrared spectrum identical to that of a ring-labeled metabolite. [b] Verified by isolation of (or failure to isolate) a metabolite with a ^{14}C-label in the methyl position which gave an infrared spectrum identical to that of a ring-labeled metabolite. [c] Identified by comparison of infrared and mass spectra with those of an authentic sample. [d] Structure verified by comparison of infrared and ultraviolet spectra with those of an authentic sample. [e] Acetyl derivative was not formed or was not characterized. [f] Structure verified by biosynthesis and isolation of a ^{35}S-labeled compound which gave an infrared spectrum identical to that of a ^{14}C-labeled metabolite isolated from urine.

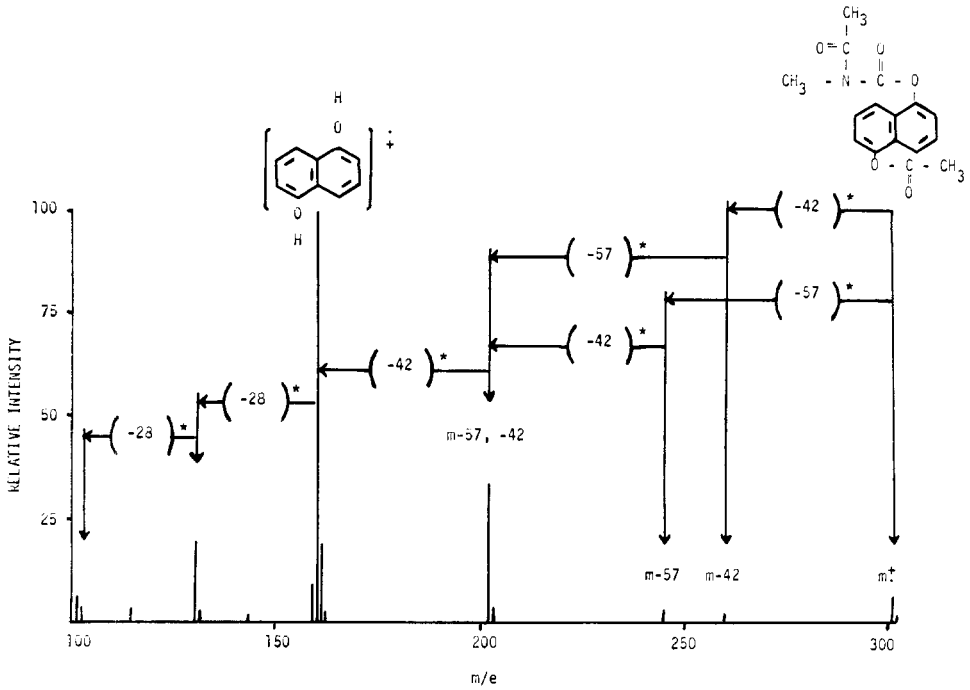


Figure 2. Mass spectrum of 1-acetoxy-5-[*N*-acetyl *N*-methyl]-carbamoylnaphthalene (referred to in the text as 5-acetoxy *N*-acetylcarbaryl). Only peaks greater than 1% are shown and * indicates a metastable loss (rearrangement)

Evaporation of the aqueous phase to dryness and extraction of the residue with methanol were important steps in the purification of the polar urinary metabolites. The radiolabeled metabolites in the residue were effectively extracted by methanol (95% or more extracted); however, most of the nonradioactive material was insoluble in this solvent. The polar metabolites were separated into five radioactive fractions when quantitatively eluted from a Sephadex G-10 column with water (shown as fractions *A*, *B*, *C*, *D*, and *E* in Figure 1). With one possible exception, each fraction from the Sephadex G-10 column contained two or more radioactive metabolites and was contaminated with other urinary constituents; however, further fractionation on a Sephadex LH-20 column developed with methanol gave excellent separation and purification of most of the polar metabolites. The radioactive metabolites were quantitatively eluted from the Sephadex LH-20 column.

The C—O—C(O)—N—C side chain was intact in metabolites 3, 5, 7, 11, 12, and 15; in each case this was demonstrated by isolation of a metabolite with a ^{14}C-label in the carbonyl position and in the methyl position which gave infrared spectra identical to that of a ring-labeled metabolite (Table I). Similar studies indicated that the side chain was not present in metabolites 4, 8, 10, 13, and 14. It was not possible to conclusively demonstrate whether the side chain was intact in metabolites 2, 6, and 9 since they were absent from the urine of some chickens given the ring-labeled compound, or they were not isolated in sufficiently pure form to give characteristic infrared spectra; attempts to convert these metabolites to their acetyl derivative were unsuccessful, or the acetylation product was not characterized.

The acetylation procedure described by Paulson and Portnoy (1970) was used successfully to aid in the characterization of the ring structure of many of the metabolites. In most cases this technique replaced the polar conjugate with an acetyl group; the acetyl derivatives were purified by gas chromatography and characterized by infrared and mass spectrometry.

Mass spectrometry was useful for the characterization of the acetyl derivatives of many of the metabolites. The spectrum of 1-naphthyl acetate showed a loss of ketene to yield an ion whose mass corresponded to 1-naphthol, which is typical of phenol acetates (Budzikiewicz *et al.*, 1967). Dihydroxy and trihydroxynaphthalene acetates gave consecutive losses of ketene to yield ions whose masses corresponded to the parent hydroxy compounds. The hydroxynaphthalene ions showed consecutive losses of CO and COH as expected; however, the position of the substitution could not be determined from the spectrum.

The mass spectrum of carbaryl was typical of a carbamate (Benson and Damico, 1968; Budzikiewicz *et al.*, 1967). The primary loss was the elements of methylisocyanate to give 1-naphthol. However, the mass spectrum of *N*-acetylcarbaryl was unexpected since acetamides normally fragment with the loss of ketene, leaving the positive charge on the nitrogen (Budzikiewicz *et al.*, 1967). In this case, an initial loss of ketene was not observed. To establish the fragmentation pathway, *N*-(trideuteroacetyl)carbaryl was synthesized using hexadeutero acetic anhydride. The initial loss of 57 mass units (elements of methylisocyanate) indicated that there was a migration of the acetyl group, yielding an ion that behaved as naphthyl acetate in subsequent fragmentation. The naphthol ion (base peak) was increased one mass unit, indicating the presence of deuterium; this demonstrated that methylisocyanate was lost first, followed by the loss of ketene. This rearrangement was recently reported by Blessington (1969).

The mass spectrum of 5-acetoxy *N*-acetylcarbaryl shown in Figure 2 is representative of the spectra obtained in this work. The base peak corresponds to the hydroxynaphthalene (in this case *m/e* 160), and the difference between this mass and the mass of naphthalene (*m/e* 128) indicates the number of hydroxy groups. The number of hydroxy groups is confirmed by the consecutive losses of CO and COH (in this case *m/e* 160 to 132, 131 to 104, 103).

The principal metabolite in the urine was identified as 1-

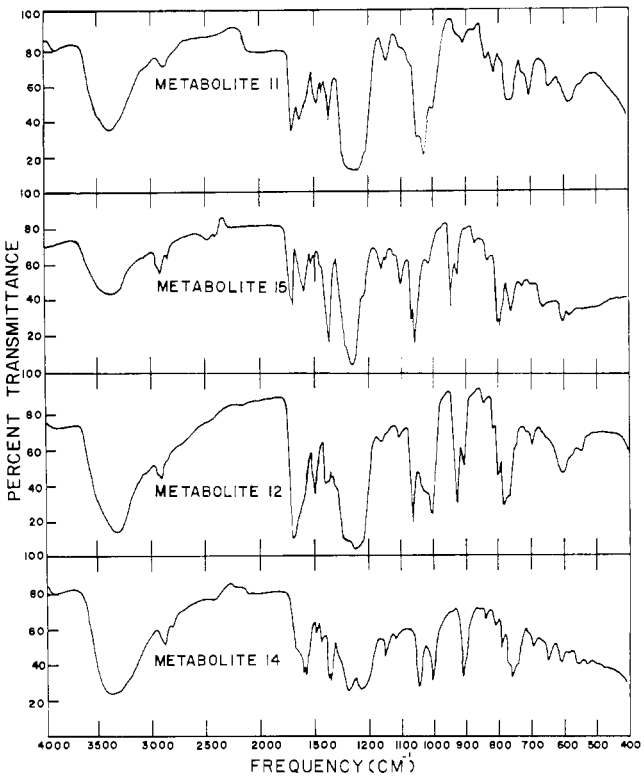


Figure 3. Infrared spectra for carbaryl metabolites 11, 12, 14, and 15 isolated from chicken urine

naphthyl sulfate by comparing its infrared and ultraviolet spectra with those of an authentic sample. An interesting observation made during these studies was that six different salt forms of this compound were separated by chromatography on a Sephadex LH-20 column developed with methanol (separation of salt forms not shown in Figure 1). The infrared spectra of the six different forms were quite similar; however, many of the absorption bands from 400 to 1500 cm.$^{-1}$ were shifted slightly, and there were some distinctly different absorption bands, particularly in the 2500- to 4000-cm.$^{-1}$ region. The sodium, potassium, and ammonium salt forms of 1-naphthyl sulfate isolated from the urine were identified by comparing their infrared spectra with those of authentic samples. The other salt forms of 1-naphthyl sulfate were not identified; however, they were readily converted to a single salt form by mass action when mixed with an excess of an appropriate inorganic salt in water. For example, when approximately 50 μg. of each of the six different forms were mixed with 10 mg. of KBr in water and then evaporated to dryness, dissolved in methanol, and chromatographed on a Sephadex LH-20 column, only one radioactive fraction was obtained. Infrared analysis of the fraction demonstrated that the six different salt forms had been converted to the potassium salt of 1-naphthyl sulfate. Similar experiments demonstrated that the six different salt forms could also be readily converted to the sodium and ammonium salt forms. Different salt forms of metabolites 4, 7, and 11 were also isolated.

Metabolite 10 was isolated in pure form and identified as 1-naphthyl-β-D-glucuronic acid by comparison of its ultraviolet and infrared spectra with those of an authentic sample.

Metabolite 11 was converted to a mixture of 4-acetoxy-*N*-acetylcarbaryl and 1,4-diacetoxynaphthalene. This, along with the observation that the entire side chain was intact (Table I), indicated that metabolite 11 was a conjugated form of 4-hydroxycarbaryl. Inspection of the infrared spectrum, as shown in Figure 3, indicated that the conjugating group was a sulfate ester (strong absorption bands at 1000 to 1060 cm.$^{-1}$ and 1200 to 1300 cm.$^{-1}$). When 4-hydroxycarbaryl, $^{35}SO_4$, and ATP were incubated with the 105,000 × G liver supernatant, a ^{35}S-labeled compound was produced, which was subsequently purified by solvent extraction and column chromatography. The ^{35}S-labeled compound gave an infrared spectrum identical to that of the ^{14}C-labeled metabolite 11 isolated from urine; from these data it was concluded that metabolite 11 was the sulfate ester conjugate of 4-hydroxycarbaryl. Metabolite 15 was characterized by similar techniques. The structure was confirmed as the sulfate ester of 5-hydroxycarbaryl by biosynthesis of the same compound (based on matching infrared spectra) from 5-hydroxycarbaryl and $^{35}SO_4$.

The entire C—O—C(O)—N—C side chain was present in metabolite 12 (Table I); however, the acetylation product was 1,5,6-triacetoxynaphthalene. The structure of the triacetyl derivative was determined by interpretation of the infrared spectra (absorption bands in the 730- to 840-cm.$^{-1}$ region indicated that the compound contained three adjacent hydrogens and two adjacent hydrogens on the aromatic nucleus), interpretation of its mass spectrum (molecular ion at *m/e* 302 and 3 consecutive losses of 42 to give a base peak of 176, which corresponded to a naphthalene triol), and synthesis of the same compound by reduction and acetylation of 5-hydroxy-1,2-naphthoquinone. Metabolite 14, which did not contain the intact side chain, was also converted to 1,5,6-triacetoxynaphthalene by the acetylation technique. The nature of the polar conjugating group was not determined for either metabolite 12 or 14; however, the infrared spectra of these two metabolites, as shown in Figure 3, suggests that they may have been sulfate ester conjugates (strong absorption bands from 1010 to 1070 cm.$^{-1}$ and 1200 to 1300 cm.$^{-1}$).

Metabolites 4 and 8 did not contain either the methyl or the carbonyl carbon (Table I). Although they were distinctly different metabolites (based on infrared spectra), they were both converted to 1,5-diacetoxynaphthalene by the acetylation procedure; thus, it was concluded that metabolites 4 and 8 were different conjugates of 1,5-naphthalenediol.

Metabolite 5 was converted to *N*-acetylcarbaryl when reacted with acetic anhydride and methanesulfonic acid. This suggested that metabolite 5 was formed by the direct conjugation of carbaryl. Knaak *et al.* (1965) have presented evidence for the possible direct conjugation of carbaryl with glucuronic acid to form 1-naphthyl methylcarbamate *N*-glucuronide and 1-naphthyl methylimidocarbonate *O*-glucuronide in the rat and guinea pig.

The polar conjugating group in metabolite 7 was not removed by the acetylation technique; however, this metabolite was readily hydrolyzed enzymatically. Characterization of the hydrolysis product indicated that metabolite 7 was a conjugated form of 4-hydroxycarbaryl.

Fraction 3 appeared to be a mixture of two different metabolites since acetylation resulted in two products (*N*-acetylcarbaryl and 5-acetoxy *N*-acetylcarbaryl). Attempts to separate these metabolites by other chromatographic techniques were not successful.

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