

# WATER-SOLUBLE METABOLITES OF CARBARYL (1-NAPHTHYL N-METHYLCARBAMATE) IN MOUSE LIVER PREPARATIONS AND IN THE RAT

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## Abstract

The biliary and urinary metabolites of carbaryl, 1-naphthyl *N*-methylcarbamate, were examined in the rat using 1-[1-<sup>14</sup>C]naphthyl *N*-methylcarbamate and 1-naphthyl *N*-methyl-[<sup>14</sup>C]carbamate. Metabolites formed by incubation of 1-[<sup>14</sup>C]naphthyl *N*-methylcarbamate with supernatant from the centrifugation at 10,000 *g* of mouse liver homogenate were also examined. In each case substantial amounts of water-soluble materials present were identified as thioether amino acid conjugates. After acid treatment these conjugates had chromatographic properties consistent with their identification as *S*-(4-hydroxy-1-naphthyl)cysteine and *S*-(5-hydroxy-1-naphthyl)cysteine. Quantitative determination allowed assignment of up to 55, 15, and 24% of the metabolites in bile, urine, and liver preparations respectively as compounds affording the cysteines on acid treatment. Some acid-labile conjugates of urine and bile were estimated indirectly by reverse isotope-dilution analyses.

## I. INTRODUCTION

Carbaryl (1-naphthyl *N*-methylcarbamate, also known as Sevin) is a widely used anticholinesterase compound possessing a wide spectrum of pesticidal activity (Carpenter *et al.* 1961). The metabolism of carbaryl has been extensively studied in mammalian species (Dorough, Leeling, and Casida 1963; Dorough and Casida 1964; Knaak *et al.* 1965, 1968; Leeling and Casida 1966; Hassan, Zayed, and Abdel-Hamid 1966; Dorough 1967; Oonithan and Casida 1968). Leeling and Casida (1966) identified 1-naphthyl *N*-(hydroxymethyl)carbamate, 4-hydroxy-1-naphthyl *N*-methylcarbamate (4-hydroxycarbaryl), 5-hydroxy-1-naphthyl *N*-methylcarbamate (5-hydroxycarbaryl), 5,6-dihydro-5,6-dihydroxy-1-naphthyl *N*-methylcarbamate (5,6-dihydro-5,6-dihydroxycarbaryl), 1,2-dihydro-1,2,5-trihydroxynaphthalene, and 1-naphthol as metabolites of carbaryl after incubation in rat, rabbit, and mouse liver microsomal preparations. The urinary conjugates of carbaryl and its metabolites have been identified as 1-naphthyl methylimidocarbonate *O*-glucuronide and the phenolic sulphates and glucuronides of 1-naphthol and 4-hydroxycarbaryl in the rat (Knaak *et al.* 1965). However, no investigations into the nature of the biliary metabolites of carbaryl have been conducted.

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This paper presents evidence for the occurrence of amino acid conjugates of carbaryl metabolites in bile and urine of treated rats and in supernatants obtained when mouse liver homogenates were centrifuged at 10,000 *g* after incubation with carbaryl.

## II. MATERIALS AND METHODS

### (a) Chemicals

Melting points are uncorrected. 1-[1-<sup>14</sup>C]Naphthyl *N*-methylcarbamate (carbaryl I) was prepared from methyl isocyanate and 1-[1-<sup>14</sup>C]naphthol (Radiochemical Centre, Amersham, England) (Skraba and Young 1959). The purified product had m.p. 142°C and a specific radioactivity of  $2.50 \times 10^7$  disintegrations/min/mg. Thin-layer chromatography showed it to be radiochemically pure. 1-Naphthyl *N*-methyl[<sup>14</sup>C]carbamate (carbaryl II) was purchased from Radiochemical Centre, Amersham, England, and had a specific radioactivity of  $2.64 \times 10^8$  disintegrations/min/mg.

4-Hydroxycarbaryl and 5-hydroxycarbaryl were prepared from the appropriate dihydroxynaphthalene and methyl isocyanate (Dorough and Casida 1964).

*S*-(4-Hydroxy-1-naphthyl)cysteine was prepared as follows. Chlorosulphonation of methyl 1-naphthyl ether afforded 4-methoxynaphthalene-1-sulphonyl chloride (Janczewski and Bartnik 1963). 4-Methoxynaphthalene-1-sulphonyl chloride (19.5 g) dissolved in tetrahydrofuran (30 ml) was slowly added to a stirred solution of lithium aluminium hydride (5.6 g) in tetrahydrofuran (75 ml). After refluxing the reaction mixture for 3 hr, 2*M*-HCl (20 ml) was slowly added to the cooled solution. The reaction mixture was then extracted with ether and the thiol isolated by extraction with 5% NaOH solution, acidification, and re-extraction into ether. Evaporation of the solvent afforded a foul-smelling yellow oil (6.18 g) which displayed -SH absorption in its i.r. spectrum (liquid film). 4-Methoxynaphthalene-1-thiol could not be crystallized and was immediately used in the next step. Crude 4-methoxynaphthalene-1-thiol (0.681 g) was refluxed for 1.5 hr in dioxan (10 ml) containing 2-acetamidoacrylic acid (0.40 g) and piperidine (0.7 ml) (Goodman, Ross, and Baker 1958). The solid which separated from the reaction mixture on standing in a refrigerator overnight was dissolved in dilute ammonia solution, reprecipitated by adjustment of the pH of the solution to 4.0, and recrystallized from aqueous ethanol. The product, *N*-acetyl-*S*-(4-methoxy-1-naphthyl)cysteine (0.30 g), had m.p. 164.5–165.5°C (Found: C, 60.0; H, 5.4; N, 4.2; S, 10.2%.  $C_{16}H_{17}NO_4S$  required C, 60.2; H, 5.4; N, 4.4; S, 10.0%). The u.v. spectrum in absolute ethanol showed  $\lambda_{max}$  at 219 nm ( $\log \epsilon = 4.64$ ) and 308 nm ( $\log \epsilon = 3.88$ ). Peaks in the n.m.r. spectrum (60 MHz) were assigned to protons as follows:  $\delta = 2.13$  (*N*-acetate); 3.72 (*O*-methyl); 9.15 (NH); and 11.33 p.p.m. (COOH). The latter two signals disappeared after D<sub>2</sub>O treatment. Refluxing 4-methoxy-1-naphthylmercapturic acid (0.78 g) with concentrated HBr (2.0 ml) in glacial acetic acid (8.0 ml) for 3 hr under N<sub>2</sub> afforded *S*-(4-hydroxy-1-naphthyl)cysteine. The cysteine (0.12 g) separated after removal of excess acetic acid under vacuum and adjustment of the residue to pH 5 with ammonia. The cysteine, which was homogeneous by paper chromatography in systems A, B, and C [see Section II(c)], fluoresced under u.v. light (253.7 nm), gave a purple colour with ninhydrin, a positive coupling test with *p*-nitrobenzenediazonium fluoroborate, and a positive test with silver chromate reagent. Attempts to recrystallize the cysteine resulted in discoloration and decomposition.

*S*-(5-Hydroxy-1-naphthyl)cysteine was similarly prepared. Methylation of sodium 5-hydroxynaphthalene-1-sulphonate according to the procedure of Mauthner (1961) afforded sodium 5-methoxynaphthalene-1-sulphonate. Treatment of the sulphonic acid salt (36.9 g) with phosphorus oxychloride (15.0 g) (Adams and Marvel 1961) afforded 5-methoxynaphthalene-1-sulphonyl chloride (6.94 g) (Oksengendler and Gendrikov 1959). 5-Methoxynaphthalene-1-sulphonyl chloride (10.5 g) was reduced with lithium aluminium hydride (4.7 g) as described above. The product, 5-methoxynaphthalene-1-thiol, was not purified but used immediately for the final step as described above for the 4-isomer. The mercapturic acid failed to separate from the reaction mixture on standing but was isolated after acidification. Recrystallization from aqueous ethanol and methanol afforded *N*-acetyl-*S*-(5-methoxy-1-naphthyl)cysteine which had

m.p. 179–180°C (Found: C, 60.5; H, 5.8; N, 4.2; S, 9.7%.  $C_{16}H_{17}NO_4S$  requires C, 60.2; H, 5.4; N, 4.4; S, 10.0%). The u.v. spectrum in absolute ethanol had  $\lambda_{\max}$  at 210 nm ( $\log \epsilon = 4.47$ ), 236 nm ( $\log \epsilon = 4.66$ ), and 312 nm ( $\log \epsilon = 4.04$ ). The i.r. and n.m.r. spectra of the product were consistent with the assigned structure. *S*-(5-Hydroxy-1-naphthyl)cysteine was prepared from the mercapturic acid by the method described above. The product could not be characterized but was homogeneous by paper chromatography in systems A, B, and C. It fluoresced blue under u.v. light (253.7 nm), gave a purple colour with ninhydrin, a positive coupling test with *p*-nitrobenzenediazonium fluoborate, and a positive test with silver chromate reagent.

All other chemicals were from commercial sources and were purified by recrystallization or distillation when necessary.

The following enzyme preparations were used: Glusulase (Endo Laboratories) contained 100,000 units  $\beta$ -glucuronidase and 50,000 units of sulphatase activity per millilitre;  $\beta$ -glucuronidase (type II, bacterial) and arylsulphatase (type III, limpet) were obtained from the Sigma Chemical Co.

#### (b) Radioactivity Determinations

Radioactive samples were assayed for  $^{14}C$  by liquid scintillation counting using a Packard Tri-Carb scintillation spectrometer, model 3314, equipped with automatic external standardization. The scintillation fluid used consisted of 0.15% (w/v) 2,5-diphenyloxazole, 0.005% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene, and 10% absolute ethanol in toluene. Aqueous solutions and samples of bile and urine were dissolved in methanol (1.0 ml) containing 0.25M methanolic Hyamine hydroxide (0.05 ml) (Packard Instrument Co.) and scintillation solvent (10 ml) then added.

#### (c) Chromatography

The descending technique was used with Whatman grade 3 chromatography paper. The following solvent systems were employed: A, butan-1-ol–acetic acid–water (2:1:1 by vol.); B, butan-1-ol–acetic acid–acetone–water (4:2:2:1 by vol.); C, propan-2-ol–formic acid–water (65:1:34 by vol.); D, butan-1-ol–propan-2-ol–3M ammonia (2:1:1 by vol.). The following spray reagents were used: *p*-nitrobenzenediazonium fluoborate for phenolic compounds (Krishna, Dorough, and Casida 1962); ninhydrin for amino acids (Boyland, Ramsay, and Sims 1961); silver chromate for bivalent sulphur compounds (Knight and Young 1958); naphthorescein for glucuronides (Harris and MacWilliam 1954). The distribution of radioactivity on chromatograms was determined by sectioning the paper into 3-cm lengths and placing each length in a scintillation vial. After addition of methanol (1.0 ml) and 0.25M methanolic Hyamine hydroxide (0.05 ml) each vial was heated on a boiling water-bath for 5 min and then scintillation fluid (10 ml) was added.

For thin-layer chromatography 0.25-mm layers of silica gel G (E. Merck A.G.) were used.

Ion-exchange chromatography was conducted on DEAE-cellulose (Whatman product DE32) according to the procedure described by Knaak *et al.* (1965).

#### (d) Metabolic Experiments

Adult male Wistar rats (250–350 g) were used in all experiments. Rats were maintained under urethane anaesthesia while the common bile duct was cannulated, and during bile collection. Solutions of carbaryl in dimethylsulphoxide (0.25–0.5 ml), prepared immediately prior to each experiment, were administered by intraperitoneal injection, or intravenous injection into the femoral vein. Bile was collected for 6 hr. Urine was collected in Jencons all-glass Metabowl cages for 24-hr intervals up to 2 days after dosing. The volume of dimethylsulphoxide used was below the lower limit (5 ml/kg) shown by Stock, Hansen, and Fouts (1970) to stimulate aniline hydroxylase *in vitro*, and no account of any possible stimulatory effect on the oxidative metabolism of carbaryl *in vivo* was taken.

In *in vitro* experiments carbaryl I (40  $\mu$ g) was deposited on the surface of the reaction vessel by evaporation of a solution in chloroform. Then 2.0 ml of supernatant from the centrifugation at 10,000 *g* of mouse liver homogenate, which had been prepared in 0.1M phosphate buffer, pH 7.4, and diluted to a 20% (w/v) homogenate with buffer, was added and the mixture shaken

in air for 4 hr at 37°C with NADP (0.115 mg), magnesium sulphate (6.41 mg), nicotinamide (6.34 mg), glucose 6-phosphate (3.92 mg) in a total volume of 3.0 ml. Blank incubations were identical except that the mouse liver homogenate supernatant was initially heated for 30 min at 90°C. The incubation mixtures were then extracted with ether to remove unchanged carbaryl and unconjugated metabolites. Since significant conversion of radioactivity to water-soluble products was obtained, this incubation mixture was considered adequate.

(e) *Examination of Metabolites*

$\beta$ -Glucuronidase hydrolysis of bile (1.0 ml) was conducted in 0.025M phosphate buffer, pH 6.8 (6.0 ml), containing  $\beta$ -glucuronidase (2 mg). The reaction mixture was incubated for 24 hr at 37°C. Arylsulphatase hydrolysis was effected by incubation for 24 hr at 37°C of arylsulphatase (2 mg) with bile (1.0 ml) and 0.2M sodium acetate-acetic acid buffer, pH 5.0 (6.0 ml). Glusulase hydrolyses were also effected in acetate buffer, pH 5.0 (6.0 ml), for 24 hr at 37°C using 0.1 ml of enzyme preparation.

Acid hydrolyses were effected by heating solutions adjusted to an acidity of 3N with hydrochloric acid for 3 hr. In all hydrolyses the non-polar metabolites were isolated by ether extraction.

In isotope dilution analyses unlabelled material was added to the urine or bile sample at the appropriate stage in the analytical procedure. The compound was reisolated from the ether extract and recrystallized to constant melting point and specific radioactivity. In analyses for 1-naphthol, the isolated 1-naphthol was chromatographed on a Florisil (Floridin Co.) column. After elution with ether-light petroleum, b.p. 60–80°C (1:1 v/v), the 1-naphthol was converted to 1-naphthyl *p*-nitrobenzoate before recrystallization.

Raney nickel desulphurizations were carried out in neutral solution. Aqueous solutions (10 ml) from acid hydrolysis were neutralized with NaHCO<sub>3</sub> and then refluxed with W-2 Raney nickel suspension (10 ml) (Mozingo 1955) and methanol (10 ml) for 2 hr. The reaction mixture was then filtered and extracted with ether to isolate the non-polar materials.

### III. RESULTS

The biliary excretion of radioactivity following intravenous dosing of rats with carbaryl I and carbaryl II is shown in Table 1, results being expressed as percentage

TABLE 1  
6-HR BILIARY EXCRETION OF RATS TREATED WITH  
[<sup>14</sup>C]CARBARYL BY INTRAVENOUS INJECTION

Results are shown  $\pm$  the standard deviation with the number of animals used indicated in parenthesis. Other results in parenthesis indicate the range of values obtained where only two animals were tested

Dose ( $\mu$ g)	Percentage Dose Excreted in Bile	
	Carbaryl I	Carbaryl II
100		50.3 $\pm$ 2.4 (3)
150		34.6 (33.8–35.4)
200	39.1 (38.7–39.5)	30.8 $\pm$ 3.3 (3)
250	40.1 $\pm$ 1.7 (3)	25.1 (21.9–28.2)
500		28.0 (27.7–28.2)
1000*		17.3 (11.9–22.7)

\* At this dose one-half of the treated rats died and biliary flow of the surviving rats was greatly diminished.

of administered dose. At a 50  $\mu$ g dose level 90% of the total biliary radioactivity was eliminated during the first 2 hr of collection. Higher doses of carbaryl could be

administered intraperitoneally without animal mortality. Thus 17.4% of a 5 mg intraperitoneal dose of carbaryl I was excreted in bile in 6 hr.

Since sulphate and glucuronide conjugates of carbaryl metabolites are present in urine of rats dosed with carbaryl (Knaak *et al.* 1965) enzymatic hydrolyses were performed on aliquots of radioactive bile with  $\beta$ -glucuronidase, arylsulphatase, and Glusulase (Table 2). Using bile from rats dosed with carbaryl I virtually no hydrolysis

TABLE 2

ENZYMATIC HYDROLYSES ON THE 6-HR BILE OF RATS TREATED WITH 250  $\mu$ G [ $^{14}$ C]CARBARYL BY INTRAVENOUS INJECTION

$\beta$ -Glucuronidase hydrolysis was effected by incubation of bile (1.0 ml) with 0.025M sodium phosphate buffer, pH 7.0 (6.0 ml), with  $\beta$ -glucuronidase (2 mg) for 24 hr at 37°C. Arylsulphatase and Glusulase hydrolyses were effected by incubation of bile (1.0 ml) with 0.2M sodium acetate-acetic acid buffer, pH 5.0 (6.0 ml), and arylsulphatase (2 mg) or Glusulase (0.1 ml) for 24 hr at 37°C. In all cases a second addition of enzyme was made after incubation for 12 hr. Results are means of duplicate experiments

Treatment	Percentage of Recovered Radioactivity		Recovery* (%)
	Aqueous Residue	Ether Extract	
Carbaryl I			
$\beta$ -Glucuronidase	81.6	18.4	103.1
Blank	90.5	9.5	99.4
Arylsulphatase	94.2	5.8	113.8
Blank	94.8	5.2	106.4
Glusulase	78.8	21.2	103.9
Blank	91.8	8.2	103.5
Carbaryl II			
Glusulase	93.1	6.9	82.7
Blank	97.3	2.7	86.5

\* Recovery is expressed as percentage of initial radioactivity.

occurred with arylsulphatase, about 9% occurred with  $\beta$ -glucuronidase, and about 13% occurred with Glusulase. Glusulase hydrolysis of bile from rats receiving carbaryl II converted only 4% of the radioactivity to ether-soluble materials. No 4- or 5-hydroxycarbaryl could be detected by reverse isotope dilution analyses on  $\beta$ -glucuronidase- and arylsulphatase-hydrolysed bile of rats dosed with 50  $\mu$ g carbaryl I.

Results of acid hydrolysis of bile of rats dosed with carbaryl I (Table 3) substantiated those obtained by enzyme hydrolysis. Only about 20% of the biliary metabolites were converted to ether-soluble materials on acid hydrolysis. A reverse isotope dilution analysis, after acid hydrolysis, on bile pre-extracted with ether showed that 17.4% of the total biliary metabolites was converted to 1-naphthol by this treatment.

Substantial amounts of radioactivity from urine of rats dosed intraperitoneally with carbaryl I remained ether-insoluble after 3N acid hydrolysis (Table 3). The

TABLE 3

ACID HYDROLYSIS OF URINE AND BILE OF RATS TREATED WITH CARBARYL I, AND OF INCUBATION MIXTURES OF SUPERNATANT FROM CENTRIFUGATION OF MOUSE LIVER HOMOGENATE WITH CARBARYL I

Urine, bile, or liver incubation mixtures were extracted with ether and then heated at 95°C for 3 hr after adjustment of acidity to 3N with hydrochloric acid. The reaction mixtures were then extracted with ether again. Each value represents a single experiment. i.v., intravenous administration; i.p. intraperitoneal administration

Dose (mg)	Tissue Analysed	Percentage of Recovered Radioactivity		Recovery (%)*
		Aqueous Residue	Ether Extract	
0.2 (i.v.)	Bile	80.9	19.1	66.5
		69.7	30.3	93.6†
5 (i.p.)	Bile	79.6	20.4	96.4
		71.8	28.2	76.2
5 (i.p.)	Urine	74.9	25.1	75.3
		48.2	51.8	69.9
—	Liver incubation mixture	74.3	25.7	71.7
		63.0	37.0	76.4

\* Recovery is expressed as percentage of initial radioactivity.

† In this case hydrolysis was effected after adjustment of acidity to 2N with hydrochloric acid.

acid-labile metabolites were examined by enzymatic hydrolyses of untreated urine with  $\beta$ -glucuronidase and arylsulphatase and reverse isotope dilution analyses. Diluents used were 1-naphthol, carbaryl, 4-hydroxycarbaryl, and 5-hydroxycarbaryl. The results obtained (Table 4) indicate the sum of the levels of unconjugated metabolite plus conjugate.

When 40  $\mu$ g carbaryl I was incubated at 37°C with supernatant from the centrifugation at 10,000 *g* of mouse liver homogenate, the bulk of the radioactivity was recovered in the water-soluble metabolites as shown in the following tabulation [see Section II(d) for experimental details]:

	No. of Expts.	Mean Percentage of Recovered Radioactivity		Recovery (%)
		Radioactivity		
		Aqueous Residue	Ether Extract	
Boiled enzyme	2	7.1	92.9	89.8
Test	4	96.1	3.9	86.3

Only a small proportion of these water-soluble metabolites were rendered ether-soluble by acid hydrolysis (Table 3).

TABLE 4

REVERSE ISOTOPE DILUTION ANALYSES FOR CARBARYL I METABOLITES IN ENZYME-HYDROLYSED 0-24-HR URINE OF RATS GIVEN 10 MG CARBARYL I INTRAPERITONEALLY

Results show duplicate assays on separate samples of urine.  $\beta$ -Glucuronidase hydrolysis was effected by incubation of untreated urine (1.0 ml) with 0.25M sodium phosphate buffer, pH 7.0 (6.0 ml), and  $\beta$ -glucuronidase (2 mg) for 24 hr at 37°C. Arylsulphatase hydrolysis was effected by incubation of urine (1.0 ml) with 0.2M sodium acetate-acetic acid buffer, pH 5.0 (6.0 ml), and arylsulphatase (2 mg) for 24 hr at 37°C. A second addition of enzyme was made after incubation for 12 hr. Results are the sum of the percentages of free metabolite plus glucuronide or sulphate

Compound	Expt. No.	Percentage of Urinary Radioactivity Present	
		After $\beta$ -Glucuronidase Hydrolysis	After Arylsulphatase Hydrolysis
1-Naphthol	1	10.0	3.8
	2	4.0	2.8
4-Hydroxycarbaryl	1	2.5	2.0
	2	1.6	1.6
5-Hydroxycarbaryl	1	3.0	1.4
	2	3.0	3.3
Carbaryl	2	2.0	2.0

TABLE 5

PAPER CHROMATOGRAPHY USING SOLVENT SYSTEM A\* OF URINE AND BILE OF RATS TREATED WITH CARBARYL OR CARBARYL I, AND OF INCUBATION MIXTURES OF SUPERNATANT FROM CENTRIFUGATION OF MOUSE LIVER HOMOGENATE WITH CARBARYL I

Bile and urine were applied directly to Whatman grade 3 chromatography paper. The incubation mixture was initially extracted with ether before application

Dose (mg)	Tissue Analysed	Detection Method*	$R_F$ Values
Various (i.v.)	Bile	Radioactivity	0.42, 0.49, 0.61
5 (i.p.)	Bile	Fluorescence	0.41, 0.48
25 (i.p.)†	0-24 hr urine	Naphthoresorcinol	0.34
25 (i.p.)†	24-48 hr urine	Radioactivity	0.52, 0.67, 0.82, 0.91
		Silver chromate	0.66, 0.79
	Liver incubation mixture	Radioactivity	0.45, 0.54

\* See Section II(c) for details.

† Dose given daily for 2 days.

That the metabolites, which were not converted to ether-soluble compounds by acid hydrolysis, might be present as thioether amino acid conjugates was suggested by the fact that naphthalene is converted *in vitro* to thioether amino acid conjugates related to *S*-naphthyl glutathione (Booth, Boyland, and Sims 1960) and that these conjugates are excreted in bile *in vivo* (Boyland, Ramsay, and Sims 1961). Paper chromatography of untreated urine and bile from rats receiving carbaryl I and of incubation mixtures of the supernatant from the centrifugation of mouse liver homogenate and carbaryl I afforded various radioactive bands (Table 5). However, except in the case of 24–48-hr urine from rats receiving 25 mg carbaryl I intraperitoneally for 2 days, no spots were obtained which gave positive silver chromate tests for thioethers. Those regions giving silver chromate tests were also radioactive.

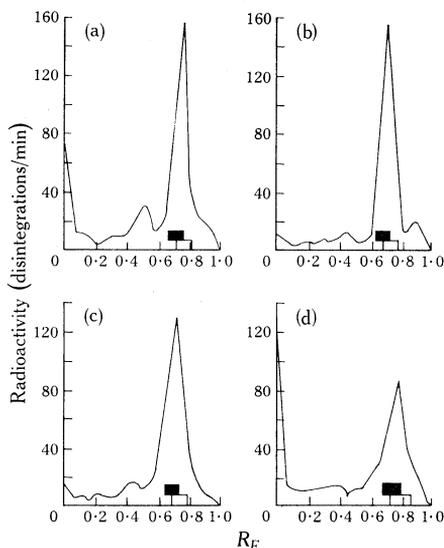


Fig. 1.—Paper chromatography of acid-hydrolysed ether-extracted urine (*c, d*) and bile (*b*) of rats dosed with carbaryl I and of acid-hydrolysed, ether-extracted supernatant from centrifugation at 10,000 *g* of mouse liver homogenate after incubation with carbaryl I (*a*). For (*a, b, c*) the developing solvent was butan-1-ol–acetic acid–water (2:1:1 v/v), and for (*d*) propan-2-ol–formic acid–water (65:1:34 v/v) was used. Whatman grade 3 chromatography paper was used with descending technique. ■  $R_F$  value of *S*-(5-hydroxy-1-naphthyl)cysteine. □  $R_F$  value of *S*-(4-hydroxy-1-naphthyl)cysteine.

When, however, the aqueous residues from acid hydrolysis of urine, bile, and liver homogenate incubation mixtures were chromatographed only one major radioactive spot occurred (Fig. 1), and this coincided with the standards, *S*-(4-hydroxynaphthyl)cysteine and *S*-(5-hydroxynaphthyl)cysteine. The two standards could not be separated from one another (see tabulation below):

Compound	$R_F$ Value		
	Solvent A	Solvent B	Solvent C
<i>S</i> -(4-hydroxy-1-naphthyl)cysteine	0.76	0.76	0.77
<i>S</i> -(5-hydroxy-1-naphthyl)cysteine	0.71	0.71	0.75

and decomposed when chromatographed using an ammonia-containing developing phase (solvent D), affording silver chromate-positive regions with  $R_F$  values 0.25

and 0.43. When the aqueous residue from acid-hydrolysed bile was chromatographed using solvent D two radioactive spots were also obtained at the same  $R_F$  values.

The amount of radioactivity of bile, urine, and liver homogenate incubation mixture which co-chromatographed with the *S*-(hydroxynaphthyl)cysteines after acid hydrolysis was determined by sectioning of the chromatograms and radiochemical determination. Up to 55.5, 15, and 24% of the initial radioactivity of bile, urine, and liver incubation mixtures respectively could be thus accounted for. Since acid hydrolyses gave poor overall recovery of radioactivity (60–90%) and incomplete recovery of radioactivity occurred on sectioning the paper chromatograms (60–90%) these amounts are regarded as minima.

To confirm the presence of thioether conjugates, a Raney nickel desulphurization was performed on the aqueous residue of acid-hydrolysed bile from rats treated with carbaryl I. Subsequent reverse isotope dilution analysis allowed 47.1% of the total biliary radioactivity to be accounted for as 1-naphthol. Attempts to perform Raney nickel desulphurizations on acid-hydrolysed rat urine and liver homogenate incubation mixtures were less successful. It was shown that acid-hydrolysed urine from untreated rats completely inhibited the desulphurization of 4-methoxy-1-naphthylmercapturic acid by Raney nickel. Also Raney nickel desulphurization of ether-extracted, acid-hydrolysed mouse liver homogenate incubation mixtures was shown to be partially inhibited by two consecutive desulphurization treatments on the same aqueous solution, each of which led to conversion of about 30% of the water-soluble radioactivity to ether-soluble materials. Consequently, reverse isotope dilution analyses for 1-naphthol were not conducted on Raney nickel-treated, acid-hydrolysed liver incubation mixtures or urine from rats treated with carbaryl I. The amount of 1-naphthol produced by desulphurization of bile is probably a minimum because of the susceptibility of Raney nickel to inactivation. However, an examination to determine the extent of such an inactivation was not made.

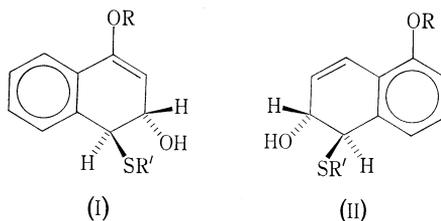
Ion-exchange chromatography on DEAE-cellulose of urine of rats dosed intraperitoneally with 5 mg carbaryl I afforded results similar to those of Knaak *et al.* (1965). Separated metabolites, whose structures had been assigned as 1-naphthyl *N*-methylimidocarbonate *O*-glucuronide, 4-hydroxycarbaryl *O*-glucuronide, 1-naphthyl glucuronide, and 1-naphthyl sulphate were hydrolysed enzymatically with the appropriate enzyme. In each case a substantial portion of the radioactivity remained in the aqueous phase after ether extraction. When the hydrolyses were effected with acid the products were not completely extracted into ether.

#### IV. DISCUSSION

The presence of thioether conjugates as carbaryl metabolites in the bile and urine of rats, and in incubation mixtures of mouse liver homogenates with carbaryl has been established. Although the complete structures of the metabolites excreted have not been determined, all are converted by acid hydrolysis to substances co-chromatographing with the *S*-(hydroxy-1-naphthyl)cysteines. Furthermore, Raney nickel desulphurization converts the acid-hydrolysed, water-soluble, biliary metabolites to 1-naphthol.

The choice of *S*-(4-hydroxy-1-naphthyl)cysteine and *S*-(5-hydroxy-1-naphthyl)cysteine as chromatographic standards was based upon the known

oxidative metabolism of carbaryl, which is converted to 4-hydroxycarbaryl, 5-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl in mammalian systems (Leeling and Casida 1966). Naphthalene, in mammalian systems, is converted to 1-naphthol, 1,2-dihydro-1,2-dihydroxynaphthalene (Boyland and Wiltshire 1953) and *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine [1-naphthyl premercapturic acid] (Boyland and Sims 1958). The formation of analogous "premercapturic acids (e.g. Ia and IIa, R = CH<sub>3</sub>NHCO or a biological modification thereof) from carbaryl



- a R = CONHCH<sub>3</sub> or biological modification  
 R' = CH<sub>2</sub>CHCO<sub>2</sub>H  
           |  
       NHCOCH<sub>3</sub>
- b R = CONHCH<sub>3</sub> or biological modification  
 R' = CH<sub>2</sub>CHCONHCH<sub>2</sub>CO<sub>2</sub>H  
           |  
       NHCOCH<sub>2</sub>CH<sub>2</sub>CHCO<sub>2</sub>H  
                           |  
                           NH<sub>2</sub>

would be consistent with its oxidative metabolism and such metabolites would be excreted in urine. Related metabolites expected in bile, urine, and liver incubation mixtures have modified amino acid residues (R'); for example bile and liver incubations would afford the glutathione derivatives (Ib and IIb). Because of the complexity and possible variety of the actual metabolites no attempts were made to synthesize them.

Acid hydrolysis of the metabolites effects cleavage of the intact carbamate ester (if present), aromatization by the elimination of water, and cleavage of the  $\gamma$ -glutamyl-cysteine and cysteinylglycine amide bonds (if present as in the glutathione, Fig. 2) or the *N*-acetate as in the "premercapturic acid". These reactions do not necessarily occur in the sequence indicated. The results of Glusulase and acid hydrolysis of bile (Tables 2 and 3) unexpectedly show that more radioactivity is converted to ether-soluble material by acid treatment than by Glusulase hydrolysis. The enzyme hydrolyses glucuronides and ethereal sulphates, both of which are cleaved by acid. This discrepancy possibly arises through the elimination of an amino acid instead of water in the aromatization of the initially formed metabolite leading to 3-hydroxycarbaryl from Ib for example. Thus Boyland and Sims (1958) obtained traces of 1- and 2-naphthol on acid treatment of 1-naphthylpremercapturic acid. A second possible explanation may be the presence of *N*-glucuronide conjugates (Knaak *et al.* 1965) which are not cleaved by enzymatic treatment with  $\beta$ -glucuronidase (Axelrod, Inscoc, and Tompkins 1958).

The failure of the urinary metabolites separated by ion-exchange chromatography to undergo complete hydrolysis by acid or enzyme indicates that as separated these metabolites are not yet pure, but are probably mixtures of thioether conjugates and the metabolite claimed. Consequently results obtained by this method for carbaryl metabolism in the rat and other species need re-examination (Knaak *et al.* 1965, 1968; Knaak and Sullivan 1967; Knaak, Sullivan, and Wills 1967). The amounts of 1-naphthyl glucuronide and 1-naphthylsulphate present in 0–24-hr urine (Table 4) are lower than levels of 11 and 16% respectively obtained by Knaak *et al.* (1965). The totals we obtained for 4- and 5-hydroxycarbaryl glucuronide and sulphate agree with Knaak's results (1965) for 4-hydroxycarbaryl. Since the same amount of carbaryl is found after both enzymatic treatments, it is probably excreted unconjugated.

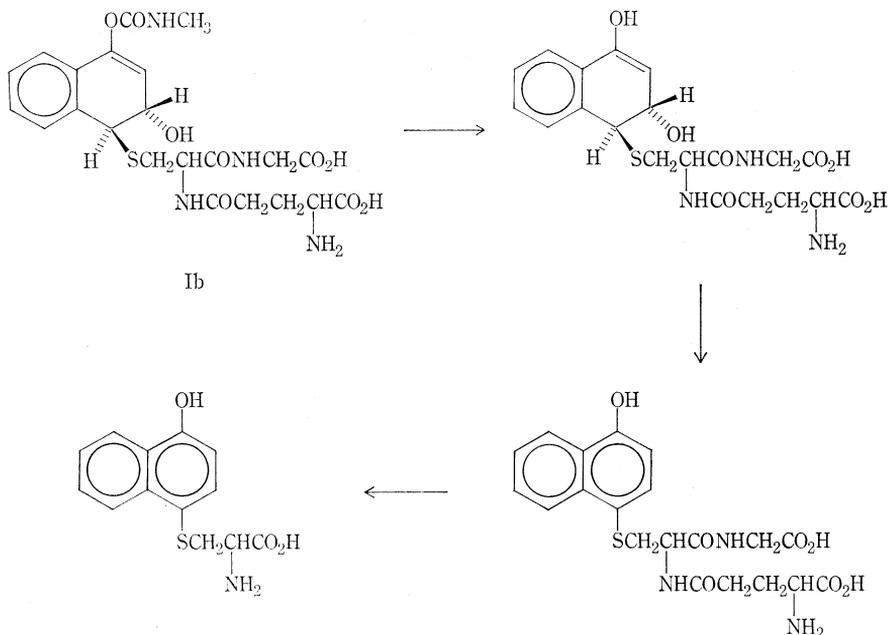


Fig. 2.—Acid hydrolysis of presumed biliary carbaryl metabolite (Ib).

Levels of biliary radioactivity obtained after dosing rats with 250  $\mu$ g carbaryl I or carbaryl II indicate that about 15% of the dose is excreted in bile after hydrolysis, while the remaining 25% possesses the intact carbamate link. Probable metabolites converted to 1-naphthol on acid hydrolysis of bile include 1-naphthyl glucuronide, 1-naphthylsulphate, 1-naphthyl *N*-methylimidocarbonate *O*-glucuronide, and 1-naphthyl *N*-(hydroxymethyl)carbamate.

Recent results have suggested that arene oxides are intermediates in the biological conversion of aromatic hydrocarbons to phenols, dihydrodiols, and glutathione and related conjugates (Jerina *et al.* 1968a, 1968b). In view of this the appearance of an aryl hydroxylated metabolite of a compound together with a dihydrodiol indicates the possible presence of thioether conjugates. This has been demonstrated with carbaryl.

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