5,6-Dihydro-5,6-dihydroxycarbaryl Glucuronide as a Significant Metabolite of Carbaryl in the Rat

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Further studies of the metabolism of carbaryl in the rat at 30 mg per kg body weight in a single oral dose have qualitatively reproduced the chromatographic profiles from urines of rats obtained by Knaak *et al.* (1965) on diethylaminoethyl cellulose columns after single oral and intraperitoneal dose studies. An attempt to verify the structure of a metabolite suspected of being 1-naphthyl methylimidocarbonate *O*-glucuronide (Knaak *et al.*, 1965) has failed to confirm the hypothesis. The principal aglycone isolated from the ether-extractable fraction of an enzyme hydrolysate of this metabolite has been identified as 5,6-dihydro-5,6-dihydroxycarbaryl. When losses and unhydrolyzed glucuronide are proportioned between this aglycone and others isolated, 10.5% of the dose is excreted as the 5,6-dihydro-5,6-dihydroxycarbaryl glucuronide calculated as carbaryl equivalents.

Carbaryl (1-naphthyl methylcarbamate) metabolism was first investigated by Carpenter *et al.* (1961). They reported that approximately 30% of the dose could be determined as 1-naphthol using a colorimetric procedure. Dorough and Casida (1964) using fortified liver microsomes found hydroxylated products of carbaryl. Knaak *et al.* (1965) using methyl, carbonyl, and naphthyl-labeled carbaryl and diethylaminoethyl cellulose chromatography analyzed the urine of rats and guinea pigs for the conjugated metabolites and found 80 to 90% of the urinary metabolites to be anionic. In addition to the glucuronide and sulfate conjugates of 1-naphthol and 4-hydroxycarbaryl (4-hydroxy-1-naphthyl methylcarbamate), these authors found a metabolite of carbaryl suspected of being 1-naphthyl methylimidocarbonate *O*-glucuronide. A chromatographically similar metabolite was found in the urines of the monkey, pig, and sheep (Knaak *et al.*, 1968) and possibly the dog (Knaak and Sullivan, 1967). Because this compound was excreted by a number of species, a program was undertaken to examine the nature and quantitative aspects of the metabolite.

METHODS

Chemicals. Carbaryl-methyl-^{14}C (0.93 mCi per mmol) and carbaryl-1-naphthyl-^{14}C (0.97 mCi per mmol) were prepared by T. E. N. Steele, Tuxedo, N. Y., as described by Knaak *et al.* (1965). Carbaryl-carbonyl-^{14}C with a specific activity of 1.5 mCi per mmol was purchased from Volk Radiochemical Co., Chicago, Ill. The samples were shown to be 99% carbaryl-^{14}C when gas chromatographed as their *N*-acetyl derivatives on a 5% SE-30 column (Sullivan *et al.*, 1967). Nonlabeled carbaryl, 5,6-dihydro-5,6-dihydroxycarbaryl, 4- and 5-hydroxycarbaryl (4- and 5-hydroxy-1-naphthyl methylcarbamate), and 1-naphthol were supplied by Union Carbide, Chemicals and Plastics Operating Division, South Charleston, W. Va.

Treatment and Handling of Animals. Naphthyl-^{14}C, methyl-^{14}C, and carbonyl-^{14}C carbaryl (0.8, 1.4, and 0.53 μCi per mg, respectively), dissolved in polyethyleneglycol 400, were administered individually by the peroral route to groups of 12, 150-g male rats at 30 mg per kg. First-day urines were collected and pooled separately according to label.

A separate study utilized two young female pigs weighing 13 to 16 kg. Naphthyl-^{14}C and *N*-methyl-^{14}C carbaryl (2.4 and 3.6 μCi per m*M*, respectively) were individually incorporated as a single dose into their diet at 500 mg/kg and first-day urines were collected according to label.

Separation of Metabolite D from other ^{14}C Urinary Metabolites. Metabolite D, the metabolite of interest, was isolated from other urinary metabolites using preparative DEAE–cellulose chromatography. Forty milliliters of urine containing the appropriately labeled rat or pig metabolite were absorbed into the top of a 5.0 × 24 cm column of DEAE–cellulose (80 g) previously equilibrated with 0.02 *M* ammonium acetate–acetic acid buffer pH 7.0, and chromatographed using a linear gradient. Twenty-four-hundred milliliters of 0.02 *M* ammonium acetate–acetic acid buffer, pH 7.0, were used in the mixing chamber and an equal volume of 0.05 *M* ammonium acetate–acetic acid buffer, pH 7.0, was used in the reservoir of the gradient device (Knaak *et al.*, 1965). Three-hundred fractions of 10 ml each were collected. During the initial work every fifth fraction was analyzed for radioactivity by liquid scintillation counting techniques in order to locate the metabolite. The desired fractions were analyzed for radioactivity, pooled (~500 ml), and the water was distilled off at 35° under reduced pressure. The metabolite, buffer, residual water, and organic impurities were removed from the distillation flask using 50 ml of methanol. The methanol was distilled from the metabolite and any residual water was removed by azeotropic distillation with acetonitrile. The metabolite and unresolved contaminants (buffer and natural products) were dissolved in 2.0 to 4.0 ml of methanol and adsorbed onto 6.0 to 7.0 g of silica gel (Grade 923, Davison Chemical Division, W. R. Grace & Co., Baltimore, Md.) and air dried. The gel containing the metabolite was then slurried in acetonitrile and added to the top of a 2.5 × 24 cm column of silica gel (80 g) previously packed in acetonitrile. Elution was accomplished using a linear gradient of acetonitrile to methanol. Six-hundred milliliters of acetonitrile were used in the mixing chamber and an equal volume of methanol was used in the reservoir of the gradient device (Knaak *et al.*, 1965). Approximately 300 fractions containing 4.0-ml volumes were collected and every fifth fraction was analyzed for radioactivity by liquid scintillation counting techniques. The fractions containing the metabolite were pooled and the solvent was removed by distillation under reduced pressure. The dried residues containing the metabolite were removed from the distillation flask and used in the following studies.

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Hydrolysis by β-Glucuronidase. The rat metabolite (naphthyl, methyl, and carbonyl labels) and the pig metabolite (naphthyl and methyl labels) present in 10 and 50 mg quantities, respectively, of the dried residue were individually dissolved in 4.0 ml of 0.1 *M* sodium acetate–acetic acid buffer, pH 4.5, and incubated with 50 mg (3000 units) of β-glucuronidase (Nutritional Biochemicals Corp., Cleveland, Ohio) for 24 hr at 37°. After incubation, the solution was adjusted to pH 7.0 and chromatographed on a 1.5 × 24 cm column of DEAE–cellulose using 300 ml of 0.02 *M* ammonium acetate–acetic acid buffer, pH 7.0, in the mixing chamber and an equal volume of 0.05 *M* ammonium acetate–acetic acid buffer, pH 7.0, in the reservoir of the gradient device (Knaak *et al.*, 1965). Approximately 150 fractions of 4.0 ml each were collected and 1.0 ml of each fraction was counted using liquid scintillation counting techniques. The neutral aglycone(s) eluting in one or two void volumes from the column was continuously extracted (24 or 48 hr) with diethyl ether from the eluting buffer at pH 7.0. At the end of the extraction period, the ether was distilled and the aglycone(s) containing a trace of water was dried by azeotropic distillation with acetonitrile.

In order to assure that work-up procedures did not alter the metabolites, a standard sample of 5,6-dihydro-5,6-dihydroxycarbaryl (Richey *et al.*, 1971) was treated in the same manner as the unknown. Ultraviolet and fluorescent analysis of the unknown and the standard sample before and after β-glucuronidase treatment indicated that no major change had taken place in the aglycone. The standard sample after extraction of the neutral aglycones from the DEAE chromatography and distillation of the ether phase had an identical ultraviolet trace as the starting material. A low level of fluorescence was detected after treatment. Thin-layer analysis indicated the presence of small amounts of 5-hydroxycarbaryl. Because known conjugates of this compound separate from metabolite(s) D, it was concluded that minor breakdown occurred during hydrolysis and treatment to produce this compound. Hydrolysis experiments with bacterial β-glucuronidase using 300 mg (15,000 units) incubated in ammonium formate buffer at pH 7.0 for 4 hr at 37° showed no evidence of 5-hydroxycarbaryl.

Acid and Base Hydrolysis. A small quantity of the residues containing the rat (10 mg) or pig metabolite (50 mg) was dissolved according to label and source in 4.0 ml of 0.05 *N* HCl or 0.05 *N* NaOH.

The metabolite in base was heated for 2.0 hr at 80° while the metabolite in acid was heated for 3.0 hr at 100°. At the end of the heating period the solutions were cooled, adjusted with acid or base to pH 7.0, and chromatographed on DEAE–cellulose, as described in the section on hydrolysis using β-glucuronidase. One milliliter of every fraction collected was analyzed for ^{14}C by liquid scintillation counting techniques. The remaining 3.0 ml from each fraction were analyzed in an Aminco–Bowman spectrophotofluorometer using a xenon lamp (American Instrument Co., part 416-992). The fractions were read at a fluorescence excitation setting of 285 nm and a fluorescence emission setting of 335 nm.

Silica Gel Chromatography of Neutrals and Aglycones. When urines or hydrolyzed glucuronides are chromatographed on a DEAE–cellulose column, materials chromatographing within one or two void volumes of the column are considered to be neutrals. The ether phase was treated as described under β-glucuronidase hydrolysis. The residues were added to 3 g of deactivated silica gel (saturated with water and air dried). The gel was dried, slurried in isooctane (2,2,4-trimethylpentane), and added to the top of a 2.5 × 24 cm column of deactivated silica gel (80 g) previously packed in isooctane. Elution was accomplished using a series of linear gradients in the order: isooctane–isopropyl ether; isopropyl ether–acetonitrile; acetonitrile–methanol. Each gradient consisted of 500 ml of the first-named solvent in the mixing chamber and an equal volume of the second solvent in the reservoir of the gradient device (Knaak *et al.*, 1965). Slightly less than 250 fractions containing 4.0-ml volumes were collected for each gradient and every fifth fraction was analyzed for radioactivity by liquid scintillation counting techniques. The fractions comprising a resolved peak were pooled and the solvent was removed by distillation under reduced pressure.

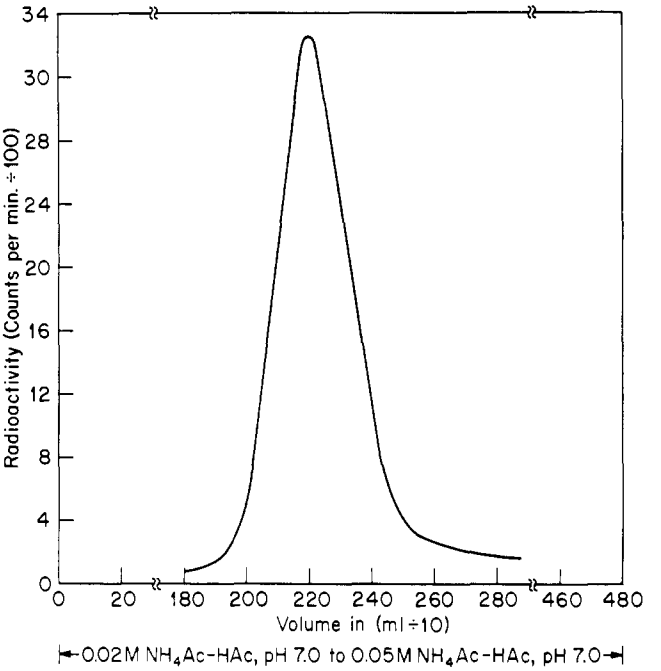

Figure 1. DEAE–cellulose chromatography of ^{14}C-labeled metabolite D on a 5.0 × 24 cm preparative column. ——, *N*-methyl, carbonyl, and naphthyl labels of carbaryl appearing in rat urine as the ^{14}C metabolite. ——, *N*-methyl and naphthyl labels of carbaryl appearing in pig urine as the ^{14}C-metabolite

Thin-Layer Analysis. The neutral aglycone(s) extract or isolated fraction was dissolved in 0.5 ml of acetonitrile and 10 to 20 μl of this solution were applied to the surface of a thin-layer plate of silica gel G (Brinkmann 5 × 20 cm precoated plates). Chromatography was carried out according to the procedure of Dorough and Casida (1964) and Leeling and Casida (1966) or in a 1:1 isopropyl ether–acetonitrile system. For identification purposes 1-naphthol, carbaryl, 4- and 5-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl were spotted on either side of the ^{14}C-labeled sample and chromatographed simultaneously. When sufficient count (greater than 5000 cpm) was available, the plates were scanned on a Nuclear–Chicago Actigraph III. When less activity was applied, radioactive materials on the plate were located by removing successive 0.5-cm areas for direct counting in scintillation vials. After removal of the labeled materials, the nonradioactive compounds were located using an aqueous spray reagent composed of 5.0 g of $K_2Cr_2O_7$ in 100 ml of 40% (v/v) H_2SO_4.

Infrared and Mass Spectroscopy. Infrared spectra were obtained on metabolite D (rat and pig) prior to hydrolysis and on the major aglycone after hydrolysis. In practice,

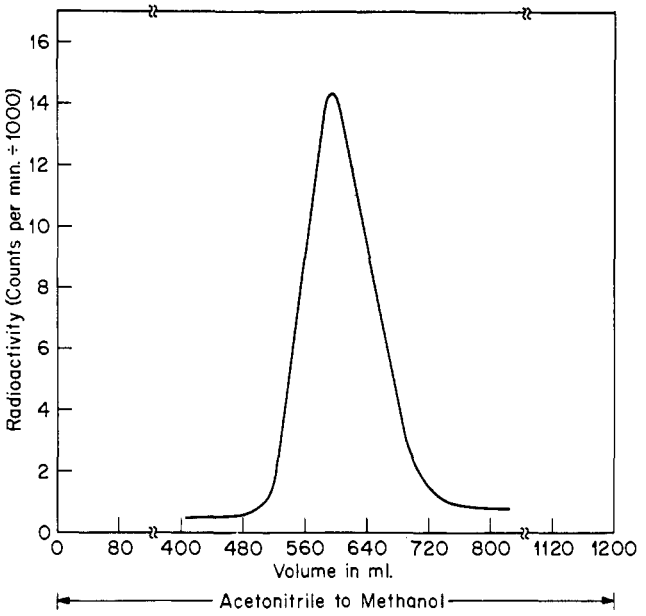

Figure 2. Rechromatography of ^{14}C-labeled metabolite D (on a 2.5 × 24 cm silica gel column)

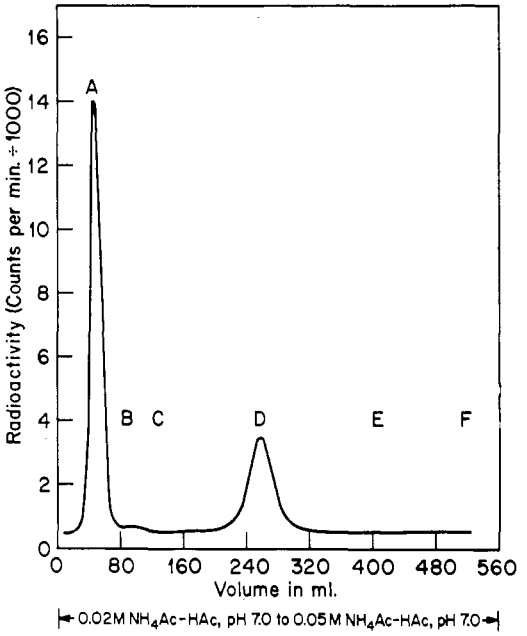

Figure 3. DEAE-cellulose chromatography of the β-glucuronidase-catalyzed hydrolysis products of metabolite D. A. Neutral aglycones; *N*-methyl and naphthyl labels, pig and rat metabolite, carbonyl label; rat metabolite. D. Unhydrolyzed metabolite; *N*-methyl and naphthyl labels, pig and rat metabolite, carbonyl label; rat metabolite

50 to 100 μg of the residues from the silica gel columns containing the ^{14}C-labeled metabolites or aglycone were mixed with approximately 10 mg of KBr. The mixture was pressed in a Beckman 18600 micropellet die to produce 1 × 5 mm pellets. The spectra were obtained on a Beckman IR-5A equipped with a beam condenser.

Mass spectra were obtained on the major aglycone and the major ether-extractable neutral component after silica gel chromatography on an AEIMS 9 instrument using ionization potentials of 14 and 70 eV and probe temperatures to 250°. The samples remaining after the initial analysis were dissolved in absolute ethanol, acidified with one drop of concentrated HCl, and allowed to stand for 3 hr. The mass spectra were then determined on the treated samples.

Table I. DEAE-Cellulose Chromatography (Figure 4) of First-Day Urines from the Rat as Carbaryl Equivalents

Naphthyl-^{14}C

Peak	Tentative identity	% Applied[a] radioactivity	% Dose
ABC	Neutrals	11.5	7.8
D	5,6-Dihydro-5,6-dihydroxycarbaryl glucuronide + unknowns	37.2	25.3
E	4-Hydroxycarbaryl glucuronide	6.8	4.6
F	Naphthyl glucuronide	11.3	7.7
G	4-Hydroxycarbaryl sulfate	6.0	4.1
H	Naphthyl sulfate	9.4	6.4
I	Unknown	...	...
J	Unknown	4.0	2.7

N-Methyl-^{14}C

Peak	Identity	% Applied[a] radioactivity	% Dose
ABC	Neutrals	28.0	19.6
D	5,6-Dihydro-5,6-dihydroxycarbaryl glucuronide + unknowns	44.0	30.8
E	4-Hydroxycarbaryl glucuronide	5.0	3.5
F	Naphthyl glucuronide	1.0	0.7
G	4-Hydroxycarbaryl sulfate	4.0	2.8
H	Naphthyl sulfate	...	...
I	Unknown	...	...
J	Unknown	...	...

[a] Calculated as percentage of the radioactivity applied to DEAE-cellulose column.

RESULTS

The metabolite(s) of interest, peak D from Knaak *et al.* (1965) and from Figure 1 and referred to as metabolite(s) D, is readily separated on DEAE-cellulose from other metabolites of carbaryl in rat and pig urine. Figure 1 illustrates the position and shape of the peak as eluted from the 5 × 24 cm DEAE-cellulose column. Regardless of the source and position of the label, the metabolite(s) chromatographed as illustrated. Metabolite(s) D was then chromatographed on a 2.5 × 24 cm silica gel column using an acetonitrile to methanol gradient. Figure 2 gives the results obtained with metabolite(s) D regardless of label or source.

β-Glucuronidase treatment of metabolite(s) D from the rat (naphthyl, carbonyl, and methyl labels) and from the pig (naphthyl and methyl labels) resulted in the formation of neutral aglycones (Figure 3). Peak A (neutral aglycones) in this figure represents approximately 90% of the recovered radioactivity for all samples. Peak D is the unhydrolyzed metabolite.

First-day urines from rats which were dosed at 30 mg/kg of naphthyl-^{14}C or methyl-^{14}C carbaryl as a single dose were collected and pooled. The urines were analyzed by the method of Knaak *et al.* (1965) using DEAE-cellulose chromatography. The results of these studies are given in Figure 4 and Table I and percentage of the radioactivity applied to the column and as percentage of dose (calculated by multiplying the above percentage by percentage of dose in first-day urine). First-day urines contained 68% of the dose as naphthyl-^{14}C and 70% of the dose for methyl-^{14}C studies.

Peak ABC in Figure 4 consists of a mixture of neutrals

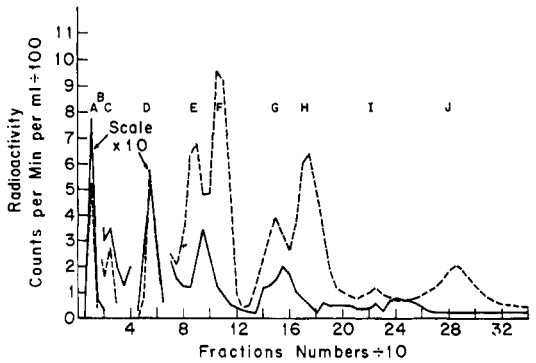

Figure 4. DEAE-cellulose chromatograph of the rat urinary metabolites of carbaryl-^{14}C. A 1.5 × 24 cm column of DEAE-cellulose was used. Gradient elution program: 0.01 *M* Tris HCl buffer, pH 7.5, to 0.05 *M* Tris HCl buffer, pH 7.5. 0.05 *M* Tris HCl buffer, pH 7.5 to 0.1 *M* Tris HCl buffer, pH 7.5. —— *N*-methyl-^{14}C. - - - - naphthyl-^{14}C

which represented 7.8 and 19.6% of the dose for the naphthyl and methyl labels, respectively. Peak D is (metabolite D) the glucuronide of interest and determined to be 25.3 and 30.8% of dose for the naphthyl and methyl labels, respectively. Peak E was designated 4-hydroxycarbaryl glucuronide (Knaak *et al.*, 1965). Values obtained for this peak were 4.6 and 3.5% for the naphthyl and methyl labels, respectively. Peak F (naphthyl glucuronide) (Knaak *et al.*, 1965) represented 7.7% of the dose for the naphthyl and 0.7% for the methyl label. Peak G (4-hydroxycarbaryl sulfate) (Knaak *et al.*, 1965) was found to represent 4.1 and 2.8% of the dose for the naphthyl and methyl labels, respectively. Addition of the G and E peaks gives total values for the designated metabolites containing 4-hydroxycarbaryl as 8.7 and 6.3% of the dose for the naphthyl and methyl labels, respectively. Peak H (naphthyl sulfate) (Knaak *et al.*, 1965) represented 6.4% of the dose for the naphthyl label, while the methyl label was slightly above background count and was too low to be quantitated. Naphthyl conjugates, the sum of the peaks F and H, were found to be 14% of the dose for the naphthyl label. With the methyl label, 0.7% of the dose was found to chromatograph in these regions. Peaks I and J are minor unknowns.

Figure 5 gives the chromatographic profile obtained by silica gel chromatography of the ether-extractable neutrals, ABC from DEAE-cellulose chromatography, from urines, and also from β-glucuronidase hydrolysis of metabolite D. Table II gives the quantitative data obtained. For peaks ABC from urines the data are reported as percentage radio-

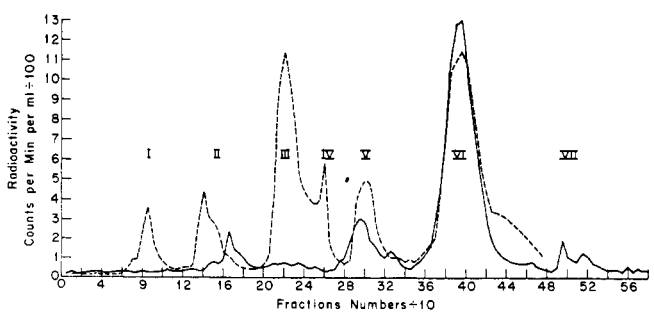

Figure 5. Chromatographic profile on silica gel of ether-extractable neutrals (ABC) from urines and hydrolysates of metabolite D. Fraction gradient elution program: 0–250, isooctane to isopropyl ether; 250–500, isopropyl ether to acetonitrile; 500–, acetonitrile to methanol; ------, neutrals in urine (peak ABC, Figure 9); ——, aglycones (peak ABC, Figure 3)

Table II. Silica Gel Chromatogram of Ether-Extractable Neutrals (Figure 5) as Naphthyl-^{14}C Carbaryl Equivalents Obtained from DEAE-Cellulose Chromatography of First-Day Urines from the Rat

Peak	% Applied[a] activity	% Neutrals[b]	% Dose
I	3.3	2.0	0.2
II	6.8	4.1	0.3
III	18.5	11.3	0.9
IV	2.5	1.5	0.1
V	7.9	4.8	0.4
VI	29.2	17.8	1.4

Silica Gel Chromatography of Ether-Extractable Neutrals (Figure 5) Naphthyl-^{14}C Carbaryl Equivalents from Glucuronide Hydrolysis with β-Glucuronidase

Peak	% Applied[a] activity	% Original glucuronide[c]	% Dose
I	...	...	...
II	6.2	2.7	0.7
III	11.0	5.0	1.3
IV			
V	12.2	5.5	1.4
VI	61.1	28.0	7.0
VII	7.0	3.2	0.8

[a] Calculated as percentage of radioactivity applied to the silica gel column. [b] Calculated as percentage of neutrals (peak ABC) from DEAE-cellulose column. [c] Calculated as percentage of metabolite(s) D from DEAE-cellulose column.

activity applied to the column, percentage of neutrals, and percentage of the dose recovered. For the neutral aglycones from the hydrolysis of metabolite D, the data are reported as the percentage of applied radioactivity, the percentage of the original glucuronide recovered, and the percentage of dose recovered. Each number or peak designation may represent more than one component. In both cases Peak VI is the largest fraction representing a significant component in the ether extract both free in urine and as an aglycone of the glucuronide. The percentage of dose from neutrals in urine was calculated by multiplying the 29.2% of applied activity by 61% extractables to give percentage of neutrals. It was then multiplied by 7.8% (neutrals as percentage of dose) to give percentage of dose recovered as Peak VI. When metabolite D (25.3% of the dose) was incubated with β-glucuronidase, 75% of the incubated radioactivity was recovered in the hydrolysate after removal of the enzyme. When this hydrolysate was passed over a DEAE-cellulose column, 90% was recovered as neutrals and 10% as unhydrolyzed metabolite D (Figure 3). Ether extraction of the neutral portion removed 68% of the radioactivity. When this extract was chromatographed on a silica gel column, 61.1% was recovered as peak VI (Figure 5). This amounts to 7% of the original dose being recovered as the aglycone of metabolite D. As demonstrated above, this value represents a minimum because all losses, unhydrolyzed components, etc., are discarded for each component in the calculation.

Specifically this report will identify only peak VI (Figure 5). However, tentative results on other aglycones and a discussion of the quantities of these materials also will be reported as they pertain to Figure 5 and Table II. Chromatographic regions, although not necessarily unique for one component, will be designated as peaks. As illustrated in Figure 5, peak I, which is missing from the aglycones, could contain 1,4-naphthoquinone, 1-naphthol, and/or 1,4-naphthalenediol as determined by calibration of the column with known standards. The major constituent of peak II by thin-layer analysis was tentatively identified as 1,5-naphthalenediol with

small amounts of carbaryl and 5-hydroxycarbaryl; only a trace of 1-naphthyl hydroxymethylcarbamate was detected. From the aglycones (solid line) no 1,5-naphthalenediol was detected, only a small amount of carbaryl and no 1-naphthyl hydroxymethylcarbamate. The majority of the radioactivity cochromatographed on thin-layer plate with 5-hydroxycarbaryl. Peaks III and IV which constitute a considerable percentage of the neutrals but a minor percentage of the dose are unknowns. These peaks are found only in the neutrals, although a small percentage of radioactivity is found throughout this region with the aglycones. Peak V is an unidentified component appearing in both the neutrals and aglycones. It is, however, a relatively minor percentage of the dose (<2%). Peak VI chromatographs on the silica gel column and thin layer with 5,6-dihydro-5,6-dihydroxycarbaryl as determined by the authentic standard, and will be discussed in the next section. Peak VII from Figure 5 contains at least two components and is found for all three labels. When the water phase of the neutral fractions from DEAE–cellulose chromatography of urines or hydrolyzed metabolite(s) D after extraction with ether are chromatographed using the same gradient system, peak VII is the first significant peak region obtained with the naphthyl label, verifying the quantitative extraction of those components eluting prior to peak VII.

Identification of 5,6-Dihydro-5,6-dihydroxycarbaryl. Peak VI from Figure 5 has been identified by thin-layer and silica gel column chromatography as 5,6-dihydro-5,6-dihydroxycarbaryl. In 4:1 ether–hexane (Dorough and Casida, 1964), the radioactivity has an R_f of 0.06 to 0.18. When the material is chromatographed in the (9:1) system of Leeling and Casida (1966), the radioactivity corresponds to their metabolite B and to authentic 5,6-dihydro-5,6-dihydroxycarbaryl. When chromatographed in a 1:1 isopropyl ether–acetonitrile system, the material migrates 9 cm from the origin (R_f 0.58), as does authentic 5,6-dihydro-5,6-dihydroxycarbaryl. These analyses indicated a single radioactive component.

Although a minor nonradioactive component was present in the fraction (peak VI, Figure 5) isolated from the column, the infrared spectrum obtained agreed with that of the standard 5,6-dihydro-5,6-dihydroxycarbaryl and the spectrum published by Leeling and Casida (1966). Spectra were also obtained of the original glucuronide mixture, metabolite D, from both the rat and the pig. The abundance of hydroxyl groups and impurities prevented analysis for most of the frequencies found. However, the absorption spectra in the region below 900 cm^{-1} are relatively clean. Spectra of metabolite D, the original glucuronide from the urines of the rat and pig, clearly show the loss of the 800 cm^{-1} absorption with a distinct band at 775 cm^{-1}. Because this region of the spectrum reflects the number of adjacent C–H bands on aromatic rings, by comparison with various carbaryl derivatives, it becomes apparent that ring modification has taken place on a majority of the carbaryl structures present in the metabolite mixture. The absorption spectrum of authentic naphthyl glucuronide was obtained to confirm this observation and both the 775 and 800 cm^{-1} bands are present as they are in carbaryl.

Ultraviolet absorption spectra were obtained for peak radioactive fractions from the DEAE–cellulose column using a Beckman Model DB spectrometer. The major urinary constituents such as phenyl glucuronide, which cochromatographs with peak D, are found but the increased absorption at 260 nm over controls is significant. Absorption peaks for naphthyl glucuronide (peak F), 4-hydroxycarbaryl sulfate (peak G), and naphthyl sulfate (peak H) showed dual maxima at 285 and 235 nm with the stronger absorption band at 235 nm. The 260-nm absorption band was missing from these spectra. These findings are in agreement with fluorescent spectra in which fluorescence equivalent to carbaryl is obtained for all of the larger peaks with the exception of metabolite D (Knaak *et al.*, 1965).

Mass spectra were obtained on samples isolated as peak VI in Figure 5. For both samples (peak VI, urinary neutrals, and aglycone), the low energy spectra showed the molecular ion of the compound at *m/e* 235, with peaks at 178 and 160. Mass measurements made when the spectra were determined were in agreement with theoretical for dihydro-dihydroxycarbaryl (*m/e* 235), a dihydro-dihydroxynaphthol (*m/e* 178), and a dihydroxy-naphthalene (*m/e* 160). These results are in agreement with those reported by Baron *et al.* (1969) on a carbaryl metabolite isolated from cow urine and milk. The samples remaining after the initial analysis were dissolved in absolute ethanol and acidified with one drop of concentrated HCl and allowed to stand for 3 hr. The mass spectra were then determined on the treated samples. The relative intensities of the 235 and 178 ions diminished with respect to the *m/e* 160 and the ion peak at *m/e* 217 increased as is required for hydroxycarbaryl.

A sample isolated as above, but from a different dosing program, was analyzed by mass spectrometry at Union Carbide Technical Center and was confirmed to be 5,6-dihydro-5,6-dihydroxycarbaryl, based upon calibrations with an authentic standard.

The melting point of the isolated metabolite was 160–162° as determined on a Fisher–Johns melting point apparatus. That of the authentic 5,6-dihydro-5,6-dihydroxycarbaryl was 162–164° on the same apparatus. When the remainder of the acid-treated samples from the mass spectral studies was reanalyzed by thin-layer chromatography, two radioactive spots could be detected. In the 4:1 ether–hexane system, one spot corresponded to the original aglycone (peak VI, Figure 5), while the second cochromatographed with 5-hydroxycarbaryl. Two radioactive spots were found using the 1:1 isopropyl ether–acetonitrile system. One spot migrating ahead of 4-hydroxycarbaryl and just behind the solvent front was consistent with the R_f of 5-hydroxycarbaryl. The remainder of the radioactivity was at R_f 0.58, where the original aglycone (peak VI, Figure 5) had chromatographed in this system.

Similar results have been obtained by DEAE–cellulose chromatography and by thin-layer analysis for the aglycone and the glucuronide from the urine of the pig. In all probability, a dihydro-dihydroxycarbaryl glucuronide is a significant metabolite in that animal as well.

Studies carried out with acid hydrolysis converted the metabolites to other forms, neutral and anionic. These acid studies produced, in addition to neutrals, a fluorescent peak chromatographing as peak E (Figure 4). The peak had the proper fluorescent spectrum and chromatographic position for 4- and/or 5-hydroxycarbaryl as prepared synthetically by Knaak *et al.* (1965) using fortified liver microsomes.

Base hydrolysis of metabolite(s) D led to a loss of radioactivity with both the methyl and carbonyl without the formation of new anionic species.

Knaak *et al.* (1965) designated this molecule as a glucuronide based upon chromatographic position from DEAE–cellulose in that it chromatographed with known weak glucuronides. This has been confirmed for other weak

glucuronides. Base hydrolysis helps support this conclusion in that methyl and carbonyl labels are lost but the base hydrolyzed metabolite containing the naphthyl label chromatographs with the original metabolite. Silica gel chromatography of glucuronides found in the rat urine does not adequately resolve glucuronides from each other but does resolve glucuronides from other urinary metabolites. Finally, β-glucuronidase treatment of the metabolite results in 90% or greater hydrolysis as determined by DEAE–cellulose analysis, while acid hydrolysis converts the metabolite to a new anionic compound chromatographing with a known glucuronide.

It must be concluded from these studies that conjugation is to the ring, with the majority of the chromatographic peaks being a conjugate of 5,6-dihydro-5,6-dihydroxycarbaryl. No evidence was found to support the proposed structure 1-naphthyl methylimidocarbonate *O*-glucuronide.

DISCUSSION

This study on the metabolism of carbaryl in the rat at 30 mg/kg as a single oral dose has qualitatively reproduced the chromatographic profile of urines of rats on diethylaminoethylcellulose columns of single oral and intraperitoneal dose studies obtained by Knaak *et al.* (1965). Quantitatively, the results in this study show a shift in distribution with an increase in the percentage of materials chromatographing as metabolite(s) D and a corresponding decrease in materials chromatographing as naphthyl glucuronide and naphthyl sulfate. A higher percentage of the methyl and carbonyl labels was found in the urine with first-day results in the present study showing 68, 70, and 75% of the dose for the naphthyl, methyl, and carbonyl labels, respectively. In an unpublished study in which nonlabeled carbaryl was incorporated into the diet at 15 mg/kg body wt/day, for at least 15 days, substitution of naphthyl or methyl-labeled carbaryl in one day's diet gave quantitative results duplicating the studies reported here. Peaks chromatographing as naphthyl glucuronide and naphthyl sulfate collectively accounted for 14.5% of the dose using the naphthyl label. Metabolite D in these feeding studies accounted for 28 and 30% of the dose for naphthyl and methyl labels, respectively. Thus, the single oral dose study reported here is probably representative of the results to be obtained when carbaryl is incorporated into the diet in chronic studies.

If the percentages of losses, unhydrolyzed glucuronide, etc., are distributed proportionately over all the aglycones, the 5,6-dihydro-5,6-dihydroxycarbaryl glucuronide would account for 40–50% of metabolite(s) D. The principal aglycone of metabolite(s) D on a proportionment of losses basis represents 10 to 11% of the dose as carbaryl equivalents with an additional 1.4% of the dose excreted as unconjugated 5,6-dihydro-5,6-dihydroxycarbaryl.

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Fly Control, Chronic Toxicity, and Residues from Feeding 2-Chloro-1-(2,4-dichlorophenyl)vinyl Diethyl Phosphate to Hens

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Technical grade Compound 4072 [2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate] was administered in the feed of laying hens for 52 weeks at 50 and 200 ppm. Excellent control of the housefly, *Musca domestica* L., and the blowfly, *Chrysomya megacephala* (F.), was obtained in droppings from the hens fed 50 ppm. No hen mortality occurred due to treatment but blood plasma cholinesterase activity was inhibited. Feed consumption, weight gain, shell thickness, egg weight, egg flavor, and odor were normal, but treatment affected egg production. Glc methods were developed to measure nanogram amounts of Compound 4072 and 2,2′,4′-trichloroacetophenone by phosphorus and electron capture detection, respectively. The limits of detectability in the various tissues ranged from 0.002 to 0.020 ppm for Compound 4072 and from 0.002 to 0.009 ppm for 2,2′,4′-trichloroacetophenone. No residues of Compound 4072 were detected, but low levels of 2,2′,4′-trichloroacetophenone were found in liver, fat, and egg yolk.

The oral administration of a number of insecticides over a lengthy period of time by means of treated feed was found to have little detrimental effect on poultry, yet it prevented the development of fly larvae in the droppings (Sherman *et al.*, 1972). Sherman *et al.* (1967a) found the acute toxicity of Compound 4072 to 10- to 12-day-old cockerels to be 29 mg/kg and that an admixture of 800 ppm in the feed given for 2 weeks had no detrimental effect on cockerels other than causing between 45 and 25% plasma cholinesterase inhibition. Droppings from these treated chicks were highly toxic to the larvae of four species of flies

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