The Metabolism of Carbaryl in the Rat, Guinea Pig, and Man

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The metabolic fate of methyl-C^{14}, carbonyl-C^{14}, and naphthyl-C^{14} carbaryl in the rat and guinea pig was investigated. The over-all recovery of the naphthyl, carbonyl, and methyl label was, respectively, 95, 99, and 91% of dose. Tissue residues (2 to 3% of dose) were found only in the case of methyl-labeled carbaryl. The metabolites identified were 4-(methylcarbamoyloxy)-1-naphthyl glucuronide, 1-naphthyl glucuronide, 4-(methylcarbamoyloxy)-1-naphthyl sulfate, and 1-naphthyl sulfate. Evidence is presented for the possible direct conjugation of carbaryl with glucuronic acid to form 1-naphthyl methyl carbamate *N*-glucuronide and 1-naphthyl methylimidocarbonate O-glucuronide. The assay of carbaryl, carbaryl derivatives, and metabolites in water by fluorometry was investigated in conjunction with C^{14} chromatographic studies and the method applied to the analysis of urines from men exposed to carbaryl dust. The only detectable metabolites present were 1-naphthyl glucuronide and sulfate.

THE METABOLISM of carbaryl, 1-naphthyl *N*-methylcarbamate, has been investigated in isolated systems (*5*) and in the intact animal (*3, 4*). These studies indicate that carbaryl is hydroxylated or hydrolyzed in the animal to 4- or 5-hydroxy-1-naphthyl methylcarbamate, 1-naphthyl hydroxymethylcarbamate, and 1-naphthol prior to the formation and excretion of their water-soluble conjugates. The urinary conjugates of these metabolites have not been separated and identified.

In the present study the metabolism of carbaryl has been re-examined and the urinary conjugates have been determined by chromatographic methods.

Methods

Chemicals. Carbaryl-methyl-C^{14} was prepared by reaction of freshly distilled naphthyl chloroformate (2.6 mmoles.) dissolved in 3.0 ml. of chloroform with methyl-C^{14} amine-HCl (2.3 mmoles; 0.28 mc. per mmole, Volk Radiochemical Co., Chicago, Ill.) dissolved in 2.0 ml. of water in a sealed reaction tube in the presence of K_2CO_3 (5.5 mmoles) at room temperature. The tube was shaken 4 hours, then opened and the aqueous phase extracted with chloroform. The chloroform phases were combined, dried using anhydrous Na_2SO_4, and evaporated onto 5.0 grams of Florisil. The carbaryl-Florisil mixture was added to the top of a 2.5- × 35-cm. column containing 70 grams of Florisil and carbaryl eluted according to the method of Krishna, Dorough, and Casida (*12*) to yield 0.416 gram of white, crystalline carbaryl. Van Slyke wet combustion of the labeled material indicated a specific activity of 0.231 mc. per mmole for a total activity of 0.481 mc. Isotopic dilution of 1.28 mg. of the radioactive product with 1.0002 grams of nonlabeled carbaryl followed by two recrystallizations from toluene yielded a new specific activity of 0.000295 mc. per mmole. This corresponded to a radiopurity of 100 ± 1.5%.

Carbaryl-1-naphthyl-1-C^{14} (0.2 mc. per mmole) was prepared from 1-naphthol and methyl isocyanate in the presence of pyridine according to the method of Skraba and Young (*14*). The 1-naphthol-1-C^{14} was purchased from Nuclear-Chicago Corp., Chicago, Ill.

Radioactive 1-naphthyl sulfate was prepared by treating 1-naphthol-1-C^{14} (0.6 mmole, 0.115 mc. per mmole) dissolved in 0.3 ml. of dimethylaniline dropwise with chlorosulfonic acid (0.72 mmole) in the cold according to the method of Feigenbaum and Neuberg (*6*). The reaction tube was tightly stoppered and agitated overnight at room temperature on a slowly revolving shaft. The viscous, light brown reaction mixture was treated with saturated aqueous KOH until slightly basic. The resultant white precipitate was stirred well with a spatula to ensure complete neutralization, after which the salt was triturated carefully with six 3.0-ml. portions of anhydrous ether. The residual solid was dried in vacuo at 60° C. for approximately 30 hours, yielding 0.244 gram of crude potassium 1-naphthyl-1-C^{14} sulfate with a specific activity of 0.074 mc. per mmole. A similarly prepared and repurified sample of nonradioactive sulfate exhibited the following analysis:

Analysis Calcd. for $C_{10}H_7SO_4K$: C, 45.8; H, 2.7; S, 12.2. Found: C, 45.7; H, 2.8; S, 12.1.

The nonlabeled 4- and 5-hydroxy-1-naphthyl methylcarbamates (4- and 5-hydroxy-carbaryl) were prepared by reaction of the corresponding naphthalenediol (0.1 mole) with 8.0 ml. of methyl isocyanate, 2 drops of dibutyltin diacetate, and 100 ml. of dry acetone in a pressure bottle overnight at room temperature. The reaction yielded mixtures of the corresponding biscarbamate, monocarbamate, and unreacted diol. The biscarbamates were relatively insoluble in acetone and were removed by filtration. The crude 4-hydroxycarbaryl was crystallized from boiling toluene to yield 14.2 grams (65%) of slightly impure product, m.p. 158–61° C. An analytical sample was obtained as colorless flat needles from benzene, m.p. 165–66° C. Seventeen grams of crude 5-hydroxy-carbaryl were obtained upon evaporation of the acetone filtrate. Several reprecipitations from ethyl acetate by dilution with hexane followed by repeated crystallization from toluene gave 4.0 grams of off-white crystals, m.p. 166–67° C. from toluene.

Analysis Calcd. for $C_{12}H_{11}NO_3$: C, 66.35; H, 5.10; N, 6.45. Found: 4-hydroxy-1-naphthyl methylcarbamate C, 66.52; H, 5.04; N, 6.29. 5-Hydroxy-1-naphthyl methylcarbamate C, 66.21; H, 5.15; N, 6.45.

Nonlabeled *N*-hydroxy-1-naphthyl methyl carbamate (*N*-hydroxycarbaryl) was prepared from the chloroformate of 1-naphthol and hydroxyl amine hydrochloride according to the procedure of Dorough and Casida (*5*).

Carbaryl-carbonyl-C^{14} with a specific activity of 1.6 mc. per mmole was purchased from Volk Radiochemical Co., Chicago, Ill., and was shown by thin-layer chromatography (*5*) and radioautography to be 98% carbaryl. Nonlabeled carbaryl and 1-naphthol were supplied by Union Carbide Olefins Division, South Charleston, W. Va. Naphthyl glucuronide was purchased from Bios Laboratories, Inc., New York, N. Y.

Uridine diphosphoglucuronide was purchased from Calbiochem, Los Angeles, Calif., and uniformly labeled uridine diphosphoglucuronic C^{14} acid was purchased from New England Nuclear Corp., Boston, Mass.

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Excretion of Carbaryl-C^{14}. Naphthyl-C^{14}, methyl-C^{14}, and carbonyl-C^{14} carbaryl dissolved in polyethylene glycol 400 were individually administered (orally) to rats (four animals per labeled compound) (1.28 to 3.9 mg. of carbaryl in 500 mg. of glycol) using the weighed syringe technique. Male rats (Carworth Farms-Elias stock) weighing approximately 150 grams were used and maintained on a commercial synthetic diet (Nutritional Biochemicals Corp., Cleveland, Ohio) containing 10% alphacel, 4% vegetable oil, 27% vitamin-free casein, 40% sucrose, 4% salt mixture, and vitamins in order to facilitate the collection of fecal material. The animals were individually housed in glass metabolism cages which permitted the separation and simultaneous collection of urine, feces, and respiratory CO_2. The daily urine and fecal samples from each animal were separately analyzed for C^{14} over a 7-day period. Respiratory CO_2 from each animal was trapped and analyzed for C^{14} over a 4-day period. After 7 days, the animals were sacrificed and their gastrointestinal tracts removed. The gastrointestinal tracts were frozen and stored separately from the carcasses and remaining organs to await analysis for C^{14}.

Methods for C^{14} Analysis. A Nuclear-Chicago scintillation spectrometer, Model 725, operated at 32° F. was used to detect and measure C^{14}. All counts were corrected to 100% efficiency values based on a standard curve of channel ratio *vs.* efficiency.

For counting purposes, urines were diluted 1 to 10 with water and 1.0 ml. of the diluted sample was added to 18.0 ml. of a scintillation mixture consisting of 1 part of xylene, 3 parts of dioxane, and 3 parts of methyl Cellosolve (methoxyethanol) with 1.0% (w./v.) 2,5-diphenyloxazole (PPO), 0.05% (w./v.) 1,4 bis-2-(5-phenyloxazolyl) benzene (POPOP), and 8.0% (w./v.) naphthalene (*2*). Dilution was required to reduce quench and increase sampling accuracy.

The fecal samples were dried in a vacuum oven at 60° C. and ground, and 0.5-gram quantities were weighed into cellulose bags made from dialysis tubing (*10*). One-half milliliter of a 10% (w./v.) sucrose solution was added, the sample thoroughly wetted, and the bag closed and dried in an oven at 60° C. The sucrose served as a binder for the powdered sample. A Parr double-valved oxygen bomb was utilized to combust the samples (*13*). The $C^{14}O_2$ produced was bubbled through two absorption traps (*9*) over a 30-minute period. Each trap contained 15.0 ml. of 20% ethanolamine in methyl Cellosolve (*8*). Two milliliters of the trapping reagent were used for counting purposes along with 18.0 ml. of the scintillation mixture. Each trap was assayed separately for radioactivity.

The gastrointestinal tracts, carcasses, and remaining organs were frozen and co-ground with dry ice in a Model ED-5 Thomas-Wiley mill. This was facilitated by constructing a Masonite box around the body of the mill and filling it with dry ice. The ground tissue was dried at 60° C. in a vacuum oven, reground, and prepared for scintillation counting, as were the fecal samples.

For the collection of respiratory $C^{14}O_2$, a modification of the metabolism cage sold by Delmar Scientific Laboratories, Maywood, Ill., was used. The second absorber containing Drierite and Ascarite removed the moisture and CO_2 given off by the animal. Slight negative pressure was employed on the outlet side of the second gas absorber to produce a flow of air through the system of 1.0 liter per minute. The flow rate (from high to low pressure) was controlled through the use of a differential low flow controller (Model 64BUL, Moore Products Co., Pittsburgh, Pa.) and flow gage. In operation, 50 grams of 8- to 20-mesh Ascarite were weighed into the CO_2 absorber and mixed with 15.0 grams of 10- to 30-mesh Chromosorb P (Johns-Manville). At the end of a 24-hour period, the Ascarite–Chromosorb P mixture was removed and reweighed and the difference in weight attributed to the CO_2 plus moisture absorbed. Because small quantities of moisture were absorbed, it became necessary to dry the mixture to constant weight in an oven prior to obtaining a weight for CO_2. A weighed amount of Ascarite plus Chromosorb P containing 3.0 grams of CO_2 was placed in the bottom of a 2.0-liter flask. The flask was fitted so that 5.0*N* H_2SO_4 could be added, the contents were stirred, and the liberated CO_2 was flushed with nitrogen into a series of two absorption traps (*9*), each of which contained 15.0 ml. of a 2 to 1 mixture of methyl Cellosolve and ethanolamine as trapping reagents. Two milliliters of the 2 to 1 mixture were then placed in a counting vial along with 18.0 ml. of the scintillation mixture.

The recovery of $C^{14}O_2$ from Ascarite was determined to be 73% by the direct liberation of a known quantity of $C^{14}O_2$ trapped on 22.0 grams of Ascarite. The $C^{14}O_2$ was obtained by the complete combustion of 100 mg. of benzoic-C^{14} acid (0.39 mc. per mmole) in a Parr oxygen bomb.

Urinary Metabolites of Carbaryl-C^{14} and Derivatives in the Rat and Guinea Pig. Naphthyl-C^{14}, methyl-C^{14}, and carbonyl-C^{14} carbaryl (3.0 mg. in 300 mg. of polyethylene glycol 400) were individually administered (intraperitoneal) to each of three 150-gram male rats. At this dose level and route of administration 73, 47, and 48% of the naphthyl-C^{14}, methyl-C^{14}, and carbonyl-C^{14} carbaryl equivalents, respectively, were excreted in pooled 24-hour urines.

Naphthyl-C^{14} and methyl-C^{14} carbaryl 3.0 mg. in 300 mg. of polyethylene glycol 400, were individually administered (I.P.) to each of three 200-gram guinea pigs. Eighty-five per cent of the count administered was recovered in 24-hour pooled urines.

For identification of the major metabolites of carbaryl-C^{14}, 1-naphthol-C^{14} (10 mg.), 4-hydroxycarbaryl (3.0 mg.), and *N*-hydroxycarbaryl (3.0 mg.) were administered individually (I.P.) to each of three 150-gram male rats. The compounds were administered dissolved in a minimum volume of polyethylene glycol 400. The count present in 24-hour pooled urines from 1-naphthol-C^{14}–treated animals accounted for 85% of the dose. Twenty-four-hour urines were pooled by compound and used in the chromatographic studies.

Twenty-four-hour control urines were obtained from nontreated animals. For chromatographic purposes the pooled 24-hour urines were adjusted to pH 7.5 with 1*N* HCl or NaOH and 2.0 ml. of urine containing 0.2 to 1.7 mg. of carbaryl-C^{14}, 1-naphthol-C^{14}, 4-hydroxycarbaryl, or *N*-hydroxycarbaryl equivalents were chromatographed on diethylaminoethyl-cellulose (DEAE-cellulose).

Urinary Metabolites of Carbaryl in Human Urine Specimens. Twenty-four-hour urine specimens were obtained by the Medical Department, Union Carbide Corp., from men exposed to carbaryl dust during normal packaging operations at the institute plant. Twenty-four-hour control specimens were obtained from the same men 72 hours after last exposure to carbaryl.

For chromatographic purposes the urines were adjusted to pH 7.5 with 1*N* NaOH and 2.0 ml. of urine were chromatographed on DEAE-cellulose.

Metabolism of Carbaryl-C^{14} and Derivatives by Liver Preparations. Guinea pig and rat liver microsomes were prepared according to the method of Smith and Breuer (*15*) for rabbit liver microsomes. The microsomal fractions were suspended in 7.0 and 15.0 ml. of 0.154*M* KCl, respectively, for the rat and guinea pig. One milliliter of this suspension corresponded to 1.0 gram of fresh liver. The rat and guinea pig liver homogenates (15% w./v.) were prepared in 0.25*M* sucrose using a Teflon-glass Potter-Elvehjem homogenizer at 4° C.

A typical incubation mixture consisted of 1.0 mg. of 1-naphthol-C^{14}, carbaryl-naphthyl-C^{14}, carbaryl-methyl-C^{14}, 4-hydroxycarbaryl, or *N*-hydroxycarbaryl dissolved in 0.2 ml. of polyethylene glycol 400; 1.0 mg. of uridine diphosphoglucuronic acid (UDPGA) or uridine diphosphoglucuronic-C^{14} acid (0.001 μc. per mmole); 1.0 ml. of 0.1*M* phosphate buffer, pH 8.0; and 1.0 ml. of the microsomal suspension or liver homogenate. In two studies involving carbaryl-naphthyl-C^{14} (Table II), 1.0 mg. of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) was added in addition to UDPGA. After incubation the mixture was extracted with diethyl ether (3 × 5 ml.) to remove organic-soluble materials (1-naphthol, carbaryl). The protein was removed from the reaction mixture by precipitation with ethanol. One milliliter of the ethanol-water solution and the ether phase were individually analyzed for C^{14}. The ethanol-water solution was rotary evaporated to a volume of 2.0 ml. and the metabolites in solution were chromatographed on DEAE-cellulose.

The ether phase was rotary evaporated to a volume of 0.5 ml., and the ether-solubles were chromatographed on thin-layer plates of silica gel G (*5*). Radioactive materials on the plate were located by removing 1.0-cm. squares from the plate for direct counting in scintillation vials. Identification was accomplished by comparing the R_f values of the radioactive

material with that of nonlabeled carbaryl, 1-naphthol, and 4-hydroxycarbaryl chromatographed simultaneously on the plate. The nonradioactive compounds were located using an aqueous spray reagent composed of 5.0 grams of $K_2Cr_2O_7$ in 100 ml. of 40% (v./v.) H_2SO_4.

Ion Exchange Chromatography of Urinary and Microsomal Metabolites of Carbaryl-C^{14} and Derivatives. The urinary and microsomal metabolites were applied to the top of a 1.5- × 24.0-cm. column of DEAE-cellulose prepared for chromatography in the following manner. Six grams of DEAE-cellulose (Nutritional Biochemicals Corp., Cleveland, Ohio) in 200 ml. of 0.01*M* tris(hydroxymethyl)aminomethane–HCl (tris-HCl), pH 7.5 were poured batchwise into a chromatographic column fitted with a fritted glass disk. The cellulose was allowed to settle under flow conditions produced by gravity and was further compacted by air pressure at 10 p.s.i. This process was repeated until the column was filled to a height of 24.0 cm. Prior to chromatography the packed cellulose was washed with 1.0 liter of the starting buffer to ensure pH equilibration.

The metabolites were eluted from the column by using two linear gradients as indicated in Figure 2. Volumes of 300 ml. were used in both the mixing chamber and the reservoir of the gradient device. The gradient device used in this study consisted of two polyethylene cylinders connected through the bottom with 5-mm. i.d. tubing. The gradient was taken off through the bottom of the mixing chamber. The flow rate was established using an all-Teflon microbellows pump (Research Appliance Co., Allison Park, Pa.).

Two hundred and eighty 4-ml. fractions were collected and 1.0 ml. per fraction was analyzed for C^{14} by liquid scintillation techniques. The remaining 3.0 ml. were analyzed in an Aminco-Bowman spectrophotofluorometer using a xenon lamp (American Instrument Co., part 416-992). All fractions were read at a fluorescence excitation setting of 285 mμ and a fluorescence emission setting of 335 mμ. Fractions 1 to 80 were made basic with 1 to 2 drops of 5*N* NaOH and allowed to stand overnight prior to reanalysis at a fluorescence excitation setting of 330 mμ and fluorescence emission setting of 465 mμ. When carbon-14 was not used, only an analysis by fluorescence was made.

Results

Excretion of Carbaryl-C^{14}. The excretion of an orally administered dose of 1-naphthyl-C^{14}, methyl-C^{14}, and carbonyl-C^{14} carbaryl equivalents by the rat is given in Figure 1 for three studies involving four animals per study. The average per cent recovered for the three labeled samples over a 7-day period was 94%. The excretion of carbaryl is essentially complete by the end of three days. Naphthyl-labeled carbaryl was not oxidized to $C^{14}O_2$ in the rat while methyl- and carbonyl-labeled carbaryl gave rise to 11 and 32% of the dose, respectively, as $C^{14}O_2$ for a 4-day period. The excretion of the naphthyl and carbonyl label was essentially complete with 95 and 99% of the label, respectively, being recovered. The methyl label was excreted to the extent of 88% of the administered C^{14}. Residual C^{14} was detected in the intestinal tracts, carcasses, and remaining organs of two animals amounting to 2 to 3% of dose,

Table I. Urinary Metabolites of Carbaryl-C^{14} Excreted by the Rat and Guinea Pig

Metabolites[a]		Metabolites Expressed as % of Total[b] C^{14} Recovered from Column				
		I	II	III	IV	V
A.	Unidentified neutrals	13.2	32.1	46.0	8.1	33.2
B.	1-Naphthyl methylcarbamate *N*-glucuronide	0.0	0.0	0.0	2.8	10.5
C.	Unidentified metabolite	4.2	10.5	11.4	1.6	4.8
D.	1-Naphthyl methylimidocarbonate *O*-glucuronide	26.0	45.3	31.3	12.4	30.1
E.	Unidentified metabolite	0.0	0.0	0.0	6.2	4.8
F.	4-(Methylcarbamoyloxy)-1-naphthyl glucuronide	10.3	7.8	6.3	15.6	12.4
G.	1-Naphthyl glucuronide	16.0	0.0	0.0	26.5	0.0
H.	4-(Methylcarbamoyloxy)-1-naphthyl sulfate	7.2	4.3	5.0	8.4	4.3
I.	1-Naphthyl sulfate	23.1	0.0	0.0	18.4	0.0

[a] Listed in order of elution.

[b] Rat studies: I. Carbaryl-naphthyl-C^{14}; II. Carbaryl-methyl-C^{14}; III. Carbaryl-carbonyl-C^{14}. Guinea pig studies: IV. Carbaryl-naphthyl-C^{14}; V. Carbaryl-methyl-C^{14}.

All urines examined were pooled collections made from 3 animals, 24 hr. after an intraperitoneal dose of 3.0 mg./animal.

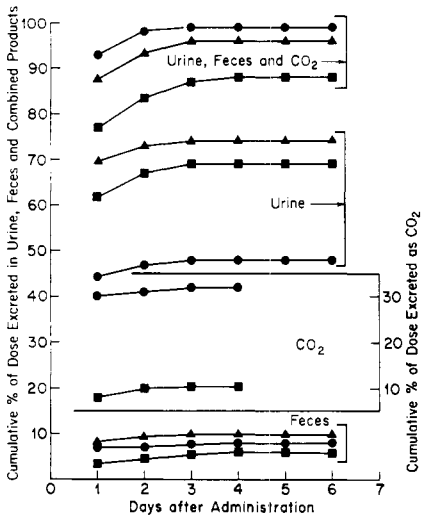

Figure 1. Excretion of carbaryl-C^{14} by the rat

▲ Average of 4 rats administered carbaryl-naphthyl-C^{14} orally at 20 mg./kg.
■ Average of 4 rats administered carbaryl-methyl-C^{14} orally at 20 mg./kg.
● Average of 4 rats administered carbaryl-carbonyl-C^{14} orally at 9.0 mg./kg.

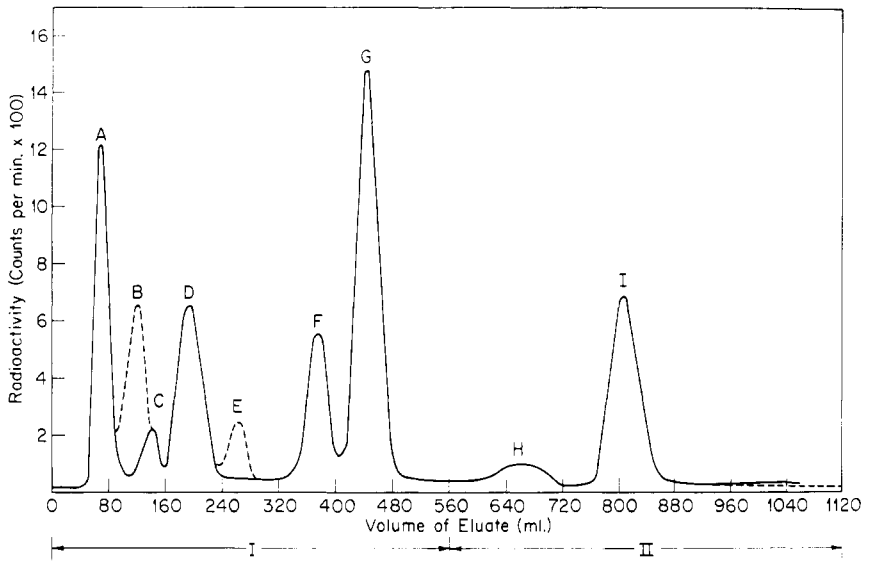

Figure 2. DEAE-cellulose chromatography of urinary metabolites of carbaryl-naphthyl-C^{14}

— Metabolites appearing in guinea pig and rat urine
– – – Metabolites found only in guinea pig urine. Metabolites identified in the text and in Table I
Gradient elution program:
I. 0.01M tris-HCl buffer, pH 7.5, to 0.05M tris-HCl buffer, pH 7.5
II. 0.05M tris-HCl buffer, pH 7.5, to 0.1M tris-HCl buffer, pH 7.5

thus bringing the over-all recovery of the methyl label to 91%.

Urinary Metabolites of Carbaryl-C^{14} and 1-Naphthol-C^{14}. The urinary metabolites of carbaryl-naphthyl-C^{14}, from the rat and guinea pig, were chromatographed on DEAE-cellulose as indicated in Figure 2. The chromatogram of Figure 2 indicates the approximate peak shape and position of each metabolite as well as their relative concentration. Table I gives the percentage of each metabolite found for naphthyl-C^{14}, methyl-C^{14}, and carbonyl-C^{14} carbaryl in the first day urine of the rat, and metabolites of naphthyl-C^{14} and methyl-C^{14} carbaryl in the guinea pig. Recoveries off the ion exchange column varied from 90 to 95% of the C^{14} applied. Variation was primarily due to difficulties in pipetting an accurately measured sample onto the column.

Metabolites in peak *A* (Table I) were not retained by DEAE-cellulose. They were eluted in one void volume and were considered to be neutrals or nonacidic metabolites. The neutrals were continuously extracted from tris buffer, pH 7.5, with diethyl ether over a 24-hour period. Diethyl ether extracted 85% of the C^{14} neutrals excreted by animals administered carbaryl-naphthyl-C^{14} and 30% from animals receiving carbaryl-methyl-C^{14}. Re-extraction of the naphthyl- and methyl-labeled neutrals at pH 2.0 recovered an additional 8.7 and 3.0% respectively, of the total. The neutrals extracted at pH 7.5 were chromatographed on thin-layer chromatoplates of silica gel G (*5*). The R_f value for 80% of the methyl-labeled C^{14} metabolites was 0.1, while 63% of the naphthyl-C^{14} metabolites possessed the same R_f value. No free carbaryl, 4-hydroxycarbaryl, 5-hydroxycarbaryl, or 1-naphthol could be found.

Metabolites *B*, *C*, *D*, *E*, *F*, and *H* were found to possess the intact C—O—C(O)N—C structure (Table I), while metabolites *G* and *I* were detected only with labeled naphthol.

Metabolite *B* was found in the pH 8.0 urines of guinea pigs administered methyl- and naphthyl-labeled carbaryl, but was not found in the pH 6.0 urines of rats administered methyl-, carbonyl-, and naphthyl-labeled carbaryl (Table I). Storage of the rat urines at pH 7.5 for 4 months (0° C.) brought about the partial conversion (50%) of metabolite *D* to metabolite *B* without the loss of the C—O—C(O)N—C structure. The rate and optimal conditions for conversion were not investigated. Metabolite *E* was found only in the urine of guinea pigs administered naphthyl- and methyl-labeled carbaryl, although small undetected amounts may be excreted by the rat.

According to Terriere, Boose, and Roubal (*16*), 1-naphthol is excreted by the rat as 1-naphthyl glucuronide and 1-naphthyl sulfate. Naphthol-C^{14} was converted in the rat to two metabolites which eluted off DEAE-cellulose in the identical positions of metabolites *G* and *I*. Naphthyl-C^{14} sulfate chromatographed similarly to metabolite *H* and metabolite *G* (a weaker acid) was tentatively identified as 1-naphthyl glucuronide.

Table II. Metabolism of Carbaryl-Naphthyl-C^{14} by Liver Preparations

	Metabolites[a], % of Total Water-Soluble C^{14}					Metabolites[a], % of Total Ether-Soluble C^{14}				% of Carbaryl-C^{14} as Water-Solubles
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	
RLM, $NADPH_2$[b]	...	...	..	...	...	6.0	83.0	8.1	2.9	...
RLM, $NADPH_2$, UDPGA	26.8	0.0	6.0	18.2	49.0	0.3	98.5	0.0	1.2	6.5
GPLM, $NADPH_2$, UDPGA[c]	30.4	0.0	0.0	20.2	49.1	0.0	92.0	0.0	8.0	17.5
RLM, UDPGA	43.0	0.0	0.0	0.0	57.0	2.5	97.5	0.0	0.0	3.0
GPLM, UDPGA	8.0	0.0	0.0	0.0	92.0	0.0	100.0	0.0	0.0	11.0
RLH, UDPGA[d]	36.0	13.0	0.0	0.0	51.0	1.5	98.5	0.0	0.0	5.0
GPLH, UDPGA[e]	1.0	0.0	0.0	1.0	98.0	0.0	100.0	0.0	0.0	10.0

[a] I. Unidentified water-soluble neutrals.
II. 1-Naphthyl methylimidocarbonate *O*-glucuronide
III. Unidentified metabolite
IV. 4-(Methylcarbamoyloxy)-1-naphthyl glucuronide
V. 1-Naphthyl glucuronide
VI. 1-Naphthol
VII. Carbaryl
VIII. 4-Hydroxy-1-naphthyl methylcarbamate
IX. Unidentified metabolites
[b] Rat liver microsomes. [c] Guinea pig liver microsomes. [d] Rat liver homogenate. [e] Guinea pig liver homogenate.

Table III. Fluorescence Characteristics of Carbaryl, Derivatives and Metabolites

Compound	Excitation Max., Mμ 1	Excitation Max., Mμ 2[a]	Fluorescence Max., Mμ	Relative Excitation 1 ÷ 2	Sensitivity Relative to Quinine Sulfate (Instrumental)
Quinine sulfate[b]	250	348	450	0.91	100.0
Carbaryl	220	285	340	0.78	51.0
1-Naphthol	230	290	465	...	20.4
1-Naphthol, pH 11.0	242	330	465	0.37	71.6
4-Hydroxy-1-naphthyl methylcarbamate	220	285	340	0.69	5.9
	232	300	480	0.58	11.4
5-Hydroxy-1-naphthyl methylcarbamate	222	290	340	0.86	4.6
	230	296	455	0.55	28.4
1-Naphthyl glucuronide	224	285	338	0.73	62.5
1-Naphthyl sulfate	218	278	335	0.61	93.0

[a] Excitation wave lengths used for maximum sensitivity. All measurements were made at concentrations of 1 μg./ml. in 0.01*M* tris buffer, pH 7.5 on an Aminco Bowman spectrophotofluorometer equipped with a xenon lamp (Aminco Bowman part 416-992) and 1/16-inch emission and excitation slits. [b] 1.0 μg./ml. in 0.1*N* H_2SO_4.

Metabolism of Carbaryl-C^{14} by Liver Preparations. Table II gives the results for a series of studies involving carbaryl-naphthyl-C^{14} and various fortified liver preparations. Rat liver microsomes fortified with a hydrogen donor ($NADPH_2$) hydrolyzed carbaryl to yield 1-naphthol and hydroxylated the naphthyl moiety of carbaryl to form 4-hydroxycarbaryl. Other products not identified were produced by the microsomes. Rat and guinea pig liver microsomes fortified with $NADPH_2$ and UDPGA converted small quantities of carbaryl-naphthyl-C^{14} to water-soluble C^{14} neutrals, metabolite *F*, and 1-naphthyl glucuronide (metabolite *G*). In systems involving microsomes, carbaryl-naphthyl-C^{14}, and UDPGA, water-soluble C^{14} neutrals and 1-naphthyl glucuronide were formed, indicating that $NADPH_2$ is required for the formation of metabolite *F*, but not for hydrolysis of carbaryl. Since 4-hydroxycarbaryl was produced by microsomes and $NADPH_2$, it was believed to be the product which gave metabolite *F* in the presence of UDPGA. When nonlabeled 4-hydroxycarbaryl was incubated with guinea pig liver microsomes in the presence of uridine diphosphoglucuronic-C^{14} acid, a C^{14}-labeled metabolite was produced having the same elution characteristics as the urinary and microsomal metabolite *F*.

Liver homogenates (rat and guinea pig) fortified with UDPGA formed water-soluble C^{14} neutrals, 4-hydroxycarbaryl glucuronide, 1-naphthyl glucuronide, and metabolite *D* from carbaryl-naphthyl-C^{14}. Metabolite *D* was also found as a product of carbaryl-methyl-C^{14}, guinea pig liver microsomes, and UDPGA. A hydrogen donor ($NADPH_2$) was not required in the formation of this metabo-

lite, indicating that the metabolite is formed through a nonoxidative pathway.

N-hydroxycarbaryl, when incubated with guinea pig liver microsomes and uridine diphosphoglucuronic-C^{14} acid, produced 1-naphthyl glucuronide-C^{14} and a second metabolite (*N*-hydroxycarbaryl glucuronide-C^{14}). The *N*-hydroxycarbaryl glucuronide eluted off DEAE-cellulose between metabolite *D* and 4-hydroxycarbaryl glucuronide. Glucuronide of *N*-hydroxycarbaryl was not a microsomal metabolite of carbaryl.

The only major urinary products not formed by the liver preparations were metabolite *H* and 1-naphthyl sulfate. Homogenates fortified with adenosine-3′-phosphate 5′-phosphosulfate (PAPS) would probably form 1-naphthyl sulfate from carbaryl.

Fluorometric Analysis of Carbaryl and Metabolites. Carbaryl does not fluoresce in the common organic solvents investigated, but will solvate in water to produce a distinctive fluorescent peak at 335 mμ. Table III gives the excitation and fluorescence maxima for carbaryl, carbaryl derivatives, and metabolites in 0.01*M* tris-HCl buffer at pH 7.5. The conditions reported are not necessarily optimal but are those under which chromatogrphic procedures are developed. Figures 3 and 4 are the chromatograms obtained from rat and guinea pig urines. Carbon-14 data from animals receiving carbaryl-naphthyl-C^{14} are included in the figures for comparison. In general, the fluorescence chromatogram was identical to the C^{14} chromatogram even though naturally occurring fluorescent products interfered in the analysis of 4-hydroxycarbaryl glucuronide (*F*) and 1-naphthyl sulfate (*I*) as shown by fluorometric analyis of control urines.

At pH 7.5 the metabolites in peak *A* are weakly fluorescent or nonfluorescent while at pH 11 some enhancement of their fluorescence is obtained. Quenching may in part be responsible for their low intensities. The fluorescence characteristics of metabolite *B* are unknown (pH 7.5 or 11). Metabolites *C* and *D* do not fluoresce at pH 7.5. However, after standing at pH 11 overnight they exhibited the fluorescent properties of 1-naphthol. For unknown reasons, the amount of 1-naphthol detected was subject to considerable variation. *N*-methyl and *N*-acetyl carbaryl (synthetic derivatives) were examined under similar conditions. *N*-Acetyl carbaryl fluoresces only after hydrolysis to 1-naphthol (pH 11) while *N*-methyl carbaryl fluoresces at pH 7.5 and 11. Carbaryl substitutions which produce a change in the hydration influence on the ring are probably responsible for the loss of fluorescence. The glucuronide of *N*-hydroxycarbaryl weakly fluoresces (1/3 the intensity of 1-naphthyl glucuronide) at pH 7.5 (335 mμ) and hydrolyzes at a

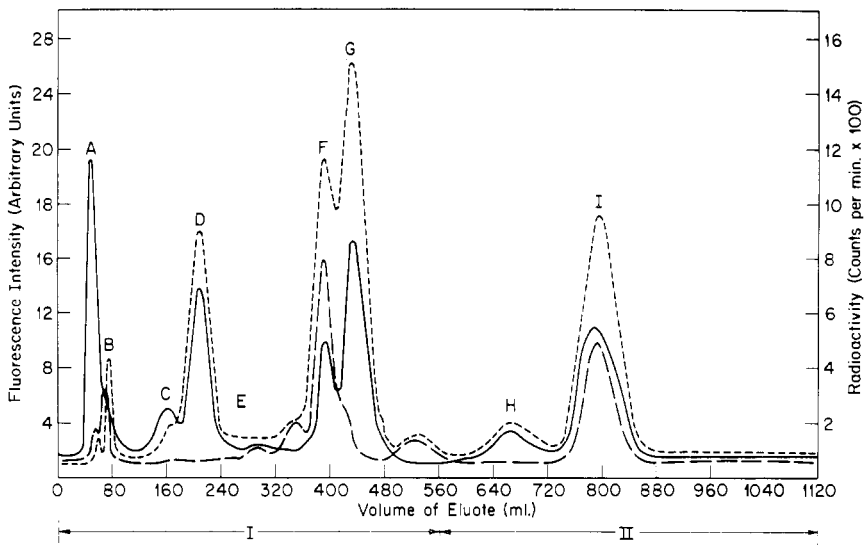

Figure 3. Fluorometric and C^{14} analysis of carbaryl-naphthyl-C^{14} metabolites present in rat urine after elution from DEAE-cellulose

—— C^{14} analysis
– – – Fluorometric analysis
— — Fluorometric analysis of natural products appearing in control urine
Gradient elution program:
I. 0.01M tris-HCl buffer, pH 7.5, to 0.05M tris-HCl buffer, pH 7.5
II. 0.05M tris-HCl buffer, pH 7.5, to 0.1M tris-HCl buffer, pH 7.5

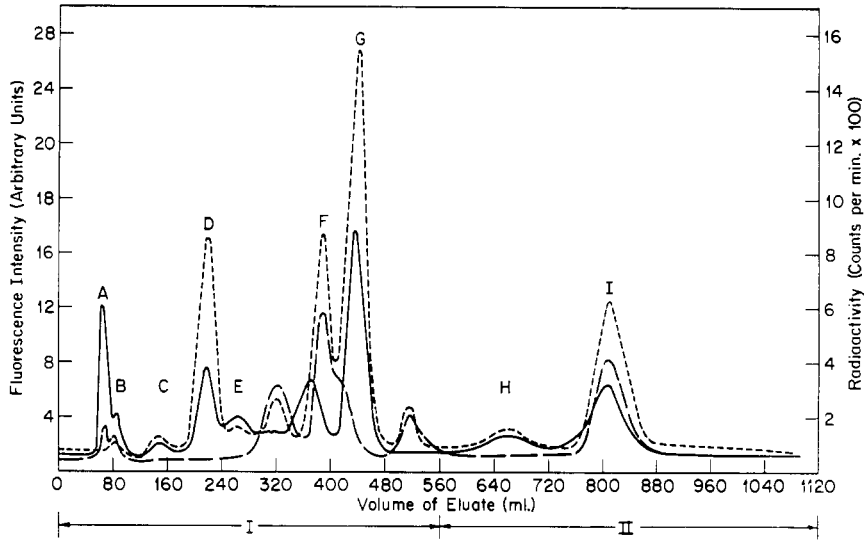

Figure 4. Fluorometric and C^{14} analysis of carbaryl-naphthyl-C^{14} metabolites present in guinea pig urine after elution from DEAE-cellulose

—— C^{14} analysis
– – – Fluorometric analysis
— — Fluorometric analysis of natural products appearing in control urine
Gradient elution program:
I. 0.01M tris-HCl buffer, pH 7.5, to 0.05M tris-HCl buffer, pH 7.5
II. 0.05M tris-HCl buffer, pH 7.5, to 0.1M tris-HCl buffer, pH 7.5

pH of 11 to give 1-naphthol as determined by thin-layer chromatography and fluorescence analysis. Metabolite *E* chromatographs in the approximate position of the glucuronide of *N*-hydroxycarbaryl, but unlike this glucuronide, the fluorescence of metabolite *E* is not enhanced at a pH of 11 (465 mμ). The identity of metabolite *E* is at present unknown. The glucuronide of 4-hydroxycarbaryl (*F*) fluoresces (335 mμ) at pH 7.5, but does not give a fluorescence maxima at 465 mμ for 1-naphthol when hydrolyzed (pH 11). Metabolite *F* probably hydrolyzes to give 4 hyd oxy-1-naphthyl glucuronide or 1,4-naphthoquinone. At pH 7.5, 1-naphthyl glucuronide fluoresces strongly (335 mμ) while no fluorescence maxima for 1-naphthol can be seen at pH 11. Naphthyl glucuronide (Bios Laboratories) cochromatographed on DEAE-cellulose with metabolite *G*, as was shown by an increase in the fluorescence of this peak, thus verifying the tentative identity of this metabolite by chromatography as well as fluorescence. Metabo-

lite *H*, containing all three labels, and 1-naphthyl sulfate fluoresce strongly at pH of 7.5 (335 mμ). Because metabolite *H* chromatographs in the position of a urinary metabolite of 4-hydroxycarbaryl, it is believed to be the sulfate of 4-hydroxycarbaryl. The order and position of elution of metabolite *H* as a sulfate of 4-hydroxycarbaryl is consistent with the facts presented for 4-hydroxycarbaryl glucuronide, 1-naphthyl glucuronide, and 1-naphthyl sulfate.

The microsomal metabolites of naphthyl-C^{14} and methyl-C^{14} carbaryl were examined by fluorescence after chromatography on DEAE-cellulose. The microsomal metabolites exhibited the same fluorescent properties as their counterparts in urine. Fluorescence analysis clearly indicated the correct order of elution of 4-hydroxycarbaryl glucuronide and 1-naphthyl glucuronide when used in combination with carbaryl-methyl-C^{14}. The glucuronides were detected by fluorescence, while C^{14} indicated that the first peak was 4-hydroxycarbaryl glucuronide and not 1-naphthyl glucuronide.

Table III gives the fluorescence intensity of 1-naphthyl glucuronide and sulfate at 1 μg. per ml. and pH 7.5 relative to quinine sulfate (1 μg. per ml.) in 0.1*N* H_2SO_4. Figure 5 depicts the chromatogram obtained with urine specimens from men exposed to carbaryl dust during normal packaging operations. Control urines were obtained 72 hours after exposure. The only detectable metabolites present were 1-naphthyl glucuronide and sulfate. The concentration of 1-naphthyl glucuronide and sulfate appearing in the urine was estimated to be 25 and 5 μg. per ml., respectively.

Discussion

Carbaryl is readily absorbed from the gastrointestinal tract and excreted. After 7 days the only detectable C^{14} residues present in the rat were from carbaryl-methyl-C^{14}. Since neither naphthyl-C^{14} or carbonyl-C^{14} residues could be detected, only the methyl moiety is incorporated in tissue (2 to 3%). Liberated naphthol is readily conjugated and excreted, while the liberated carbonyl group is disposed of as respiratory $C^{14}O_2$.

Carbaryl is metabolized in the rat and guinea pig to a series of eight or more water-soluble compounds. Forty-seven to 57% of the metabolites excreted possess the intact C—O—C(O)N—C structure, indicating that a nonhydrolytic pathway exists for carbaryl. The glucuronide of 4-hydroxycarbaryl (*F*, 10 to 15%) and metabolite *D* (12 to 26%) are the major metabolites in this group. Although the exact nature of metabolite *D* is unknown, it chromatographs on DEAE-cellulose similarly to glucuronic-C^{14} acid and 2-ethylhexanoyl-C^{14} glucuronide (*11*), and is believed to be

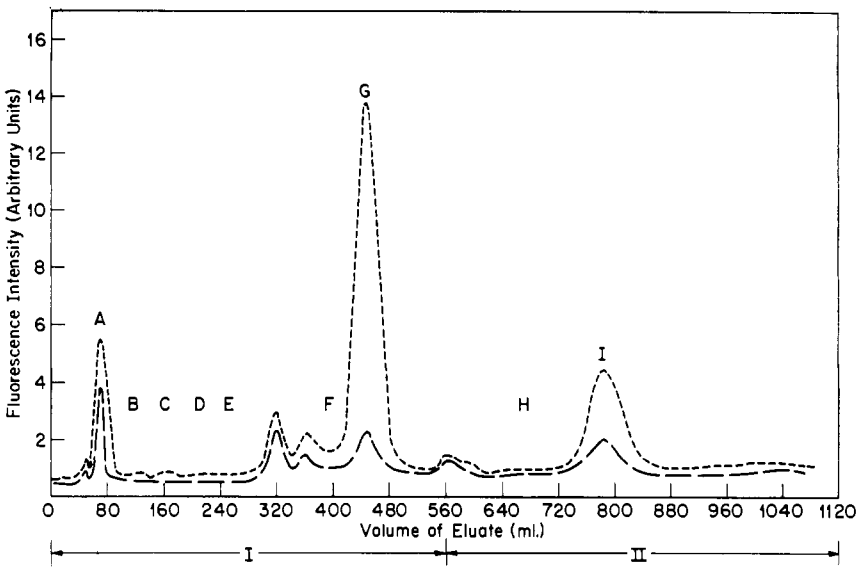

Figure 5. Fluorometric analysis of carbaryl metabolites present in urine of men exposed to carbaryl dust after elution from DEAE-cellulose

--- Fluorometric analysis
— — Fluorometric analysis of natural products appearing in control urine
Gradient elution program
I. 0.01M tris-HCl buffer, pH 7.5, to 0.05M tris-HCl buffer, pH 7.5
II. 0.05M tris-HCl buffer, pH 7.5, to 0.1M tris-HCl buffer, pH 7.5

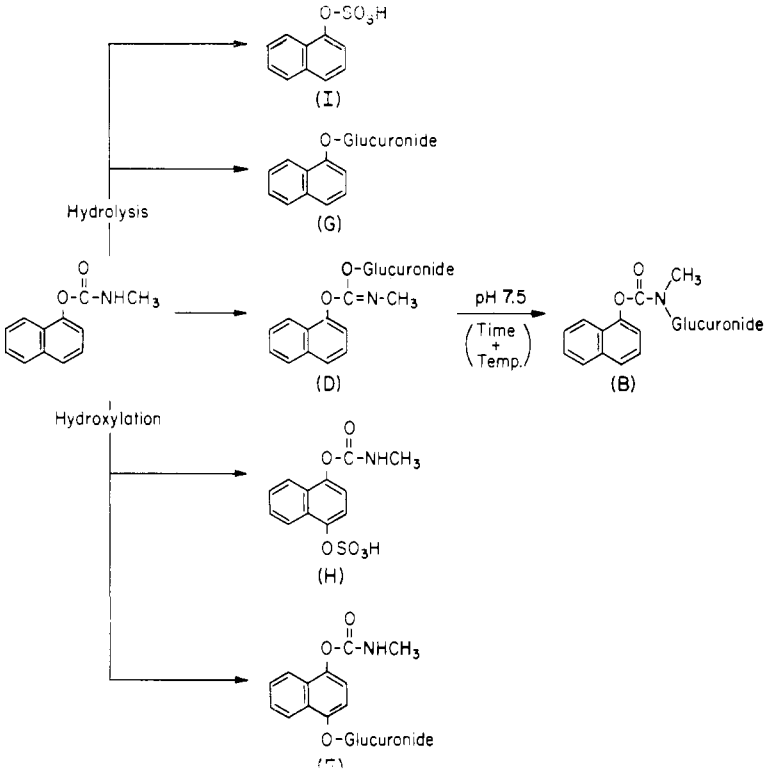

Figure 6. Metabolic path for carbaryl in the rat and guinea pig

an *O*-glucuronide of carbaryl (Figure 6) through the enol form. Carbaryl *O*-glucuronide (1-naphthyl methylimidocarbonate *O*-glucuronide) represents the enol form of the carbamate. The observed conversion of metabolite *D* to *B* may be explained on the basis of the known (*6*) irreversible rearrangement of imidocarbonates to the corresponding *N*-substituted carbamates. Metabolite B is believed to be an *N*-glucuronide of carbaryl (1-naphthyl methylcarbamate *N* - glucuronide). Bartley (*1*) has successfully synthesized 1-naphthyl ethyl *N*-methyl imidocarbonate (carbaryl *O*-ethyl) to substantiate the formation of imidocarbonates of carbaryl. This tentative assignment is supported by the difference in the fluorescent properties of 4-hydroxycarbaryl glucuronide and *N*-hydroxycarbaryl glucuronide. A glucuronide of carbaryl—

i.e. 4-hydroxycarbaryl glucuronide—conjugated through the naphthyl moiety fluoresces at pH 7.5 (335 mμ) and is nonfluorescent at pH 11 (465 mμ), while metabolite *D* (carbaryl *O*-glucuronide) fluoresces at pH 11 (465 mμ), but is nonfluorescent at pH 7.5. Conjugation of carbaryl through the *N*-hydroxy group produces a molecule that has fluorescent properties similar to carbaryl, while metabolite *D* has fluorescent properties similar to *N*-acetylcarbaryl. Metabolite *C* displays fluorescent properties similar to *D* and *N*-acetylcarbaryl. At the present time the identity of metabolite *C* is unknown.

Thirty-nine to 44% of the administered carbaryl was hydrolyzed and the liberated 1-naphthol conjugated with glucuronic and sulfuric acids. Fluorometric and chromatographic analysis of urines obtained from men exposed to carbaryl dust during packaging operations yielded additional evidence for conjugation after hydrolysis.

Fluorescence was found to be useful during radioactive studies in confirming the presence of the naphthyl moiety by direct (4-hydroxycarbaryl glucuronide, naphthyl glucuronide, 4-hydroxycarbaryl sulfate, naphthyl sulfate) or indirect (carbaryl *O*-glucuronide) analysis. Fluorescence analysis combined with established chromatographic procedure made possible the identification of the two principal metabolites of carbaryl in man.

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RESIDUES IN CITRUS

2,2-Dichloropropionic Acid Residues in Citrus Fruit from Florida Following Applications of the Herbicide

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Dalapon residues in citrus fruits from Florida following a variety of Dowpon applications ranged from 0 to 11 p.p.m. The recovery of added dalapon was 90% over the range of 0.2 to 20 p.p.m. The residual herbicide in a sample appeared to be related to several factors including the actual amount of chemical applied. A maximum residue of 5 p.p.m. may result from treatments that are recommended on the label. A study related to time between application and harvest indicates that the greatest residue in fruit is found at the time of a flush of growth of the trees. No loss of chemical was caused by a heat treatment simulating commercial pulp drying. Residues from ditch-bank treatments were 0.2 p.p.m. and lower.

THE PREVALENCE of many species of grass in areas where citrus fruit is grown causes a very difficult control problem. Bermuda (*cynodon dactylon*), bahia (*Paspalum notatum*), para (*Panicum purpurascens*), guinea (*Panicum maximum*), maidencane (*Panicum hemitomon*), and Johnson (*Sorghum halepense*) grasses are among the principal offenders, competing with the citrus trees for water and nutrients. Uncontrolled grass growth makes other pest control difficult, and adds greatly to grove maintenance problems. By the proper use of Dowpon (The Dow Chemical Co.) containing 85% of the sodium salt of dalapon (trade-mark of The Dow Chemical Co. abroad for 2,2-dichloropropionic acid), it is possible to control most grasses safely with a minimum of labor.

This study was undertaken to determine the residue of dalapon in citrus fruit from areas treated with Dowpon for control of grasses.

The data presented here were used in a petition to the U. S. Food and Drug Administration to establish a tolerance for dalapon in citrus fruits from Florida (*7*). As a result of this work, Dowpon is accepted for use in commercial orchards in Florida with a legal tolerance of 5 p.p.m. of dalapon in grapfruit, limes, tangerines, and oranges.

Experimental

Applications were made as indicated in Tables II, III, and IV. In general, each experiment consisted of treatment of plots of 2 to 6 trees replicated at least twice. A sample was taken by random selection of 4 to 10 oranges from each tree, which were combined into a single sample. When duplicate samples were collected, the process was repeated.

The samples were shipped to the