Confirmatory Isolation and Identification of a Metabolite of Carbaryl in Urine and Milk

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This study confirms the chemical nature of the major organic extractable metabolite of carbaryl in milk and urine. The data obtained with the aid of ultraviolet, infrared, mass, and nuclear magnetic resonance spectroscopy are consistent with the structure of the methylcarbamate of 5,6-dihydro-5,6-dihydroxy-1-naphthol. The compound exhibits a lower degree of toxicological hazard than carbaryl, as evidenced by anticholinesterase and fly bioassay tests, and has a very low potential as a microbial mutagenic agent. The presence of this metabolite may be qualitatively confirmed by colorimetric analysis, but no quantitative assay is available. The organic synthesis and final proof of structure are yet to be determined.

Carbaryl (1-naphthyl *N*-methylcarbamate) is degraded in mammals and plants by oxidative and/or hydrolytic mechanisms. Dorough and Casida (1964) first believed the major nonconjugated carbamate metabolite of carbaryl to be the methylcarbamate ester of 3,4-dihydro-3,4-dihydroxy-1-naphthol; Leeling and Casida (1966) later identified it as the methylcarbamate ester of 5,6-dihydro-5,6-dihydroxy-1-naphthol. This metabolite and others occur in mammalian urine, plants, and milk (cow and goat), in both the free and conjugated form. The chemical nature of the metabolite was derived by interpretation of the infrared spectrum and characterization of the products resulting from alkaline hydrolysis and acid degradation. Preliminary work on a method of analysis for the metabolite indicated that the hydrolytic product might couple with Gibbs' reagent (*N*,2,6-trichloro-*p*-benzoquinoneimine) to yield a blue complex (Dorough, 1967). Limited bioassays (housefly) and in vitro antiesterase activity, relative to carbaryl as a standard, show that the metabolite is one fiftieth as toxic to the housefly and one fifteenth as inhibitory to cholinesterase (Dorough and Casida, 1964).

This study was undertaken in an effort to confirm the structure and to evaluate and expand upon the preliminary toxicological findings.

METHODS AND MATERIALS

Carbaryl, 2.006 grams containing 3.5 mc. of ^{14}C-carbonyl-labeled carbaryl, was administered to a cross-bred Holstein cow. Details of the treatment and milk sampling were previously reported (Baron *et al.*, 1968). Urine containing radioactive residues was collected for 168 hours following treatment. In an effort to obtain larger quantities of metabolites, the cow was treated five times at weekly intervals with 10 grams of nonlabeled carbaryl, and milk and urine were collected for 24 hours. The five individual milk samples were pooled and the five urine samples were pooled, frozen, and stored.

Details of the analysis of milk were previously reported (Baron, 1968). In this study, the chloroform extract was qualitatively analyzed for the presence of metabolites with techniques of thin-layer chromatography (TLC). Silica gel G (250-micron thickness) was used as the adsorbent with ether-hexane (3 to 1, v./v.) as the developing solvent. The components were detected on the TLC plate by radioassay and/or by colorimetric analysis, after acidification and alkaline hydrolysis, with *p*-nitrobenzenediazonium fluoborate (saturated ethanolic solution) and Gibbs' reagent (0.1% *N*,2,6-trichloro-*p*-benzoquinoneimine). Urine was extracted with ethyl ether for 1 to 5 days in a liquid extraction apparatus; after the extraction, most of the ether was evaporated in a Kuderna-Danish apparatus fitted with a Snyder column. Florisil column chromatography was used for initial cleanup and separation. Seventy-five grams of Florisil was heated at 130° C. for 24 hours; it was then cooled, slurried in hexane, and packed into a 3.5-cm. (i.d.) chromatographic column. Twenty-five grams of Florisil was mixed with an aliquot of the ether extract and the mixture was dried in vacuo at 40° C. for 1 hour. The Florisil mixture was then slurried in hexane and packed on top of the column, and the excess hexane was allowed to drain before the eluting solvent was added. One liter each of 1-to-1, 2-to-1, 3-to-1, 4-to-1, and 6-to-1 (v./v.) ether-hexane mixtures were used to elute the metabolites at a flow rate of 20 ml. per minute; 20-ml. fractions were collected. The individual fractions of the radioactive urine extract were analyzed by scintillation counting techniques (Packard Instrument Co., LaGrange, Ill., Model 3365). Quantitative data were not of primary concern; the values obtained in counts per minute were used to show peak locations. The corresponding nonradioactive metabolites were collected according to the established peak locations, and the radioactive and nonradioactive fractions were pooled.

The urinary extract was further purified by silica gel column chromatography. Seventy-five grams of silica gel (E. Merck, Darmstadt, Germany; 0.05 to 0.2 mm., 70- to 325-mesh, ASTM) was slurried in hexane and packed in a 3.5-cm. (i.d.) column. An aliquot of the Florisil-purified urinary extract was added to 5 grams of silica gel, the solvent was removed by rotary evaporation at ambient temperature, and the silica gel mixture was then slurried in hexane and added to the column. The column was connected to a flow cell apparatus (Packard Instrument Co.) packed with lithium-activated glass beads and the effluent was monitored for ^{14}C through one channel of the spectrometer and a recording rate meter. The solvent system used for elution was a 6-liter linear gradient of hexane to ether flowing at a rate of approximately 1.5 ml. per minute. Sample fractions were collected at 12-minute intervals. The fractions containing the metabolite were pooled, reduced in volume by about 25%, and stored in the freezer to induce crystallization.

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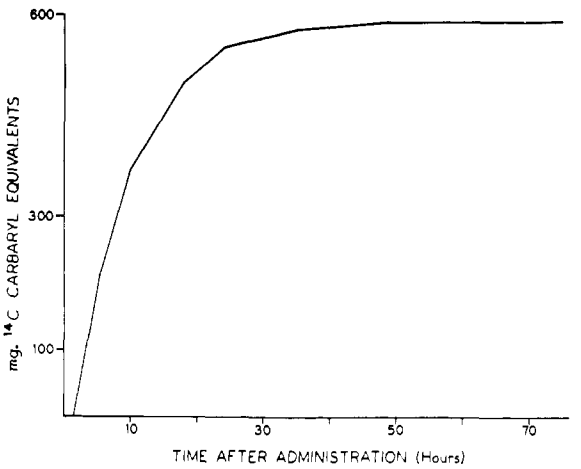

Figure 1. Cumulative urinary excretion of ^{14}C radioactivity following administration of carbonyl-^{14}C-carbaryl to a lactating cow

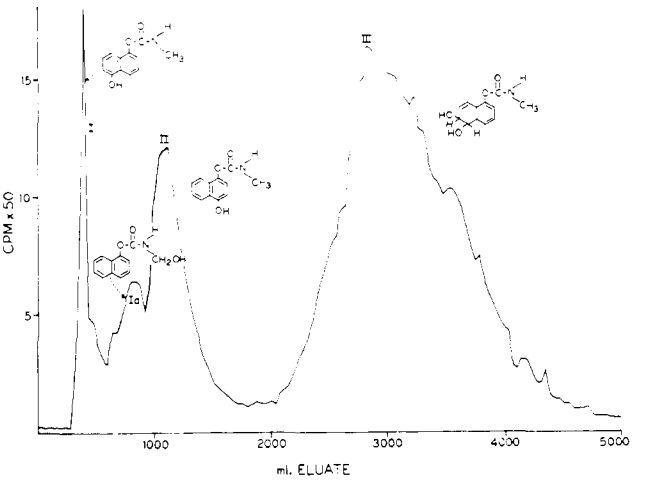

Figure 2. Florisil column chromatographic separation of urinary metabolites of carbaryl

Infrared spectra of the crystals were taken with a Beckman IR-5A using a micro KBr disk technique. Ultraviolet spectra were obtained with a Cary 14 in a 1-cm. cell against ethanol as a reference. Mass spectra were obtained with an Atlas CH-4 mass spectrometer; a combination electron impact–field ionization source and a molecular beam inlet system were used. The molecular beam inlet system, composed of a direct inlet probe, permits thermal independence between the sample temperature and ion source temperature, with the sample placed a short distance (2 mm.) from the ionization chamber. The conditions for electron impact were: electron energy, 70 e.v.; accelerating voltage, 3 kv.; magnetic scanning; ion source temperature, 240° C.; sample temperature, 55° C.; and the ion source potentials adjusted for maximum beam current. The conditions for the field ionization were: wire emitter; cathode potential, − 6 kv.; anode potential, +3 kv.; accelerating voltage, 3 kv.; magnetic scanning; ion source temperature, 200° C.; sample temperature, 68° C.; and the ion source potentials adjusted for maximum beam current. High resolution mass spectra were obtained with a CEC 21110 mass spectrometer.

NMR spectra were taken with saturated solutions (about 5%) in CH_3OD and in $(CD_3)_2CO$ containing 10% $(CH_3)_2CO$. The instruments used were Varian A-60 and HA-100 spectrometers, in combination with the C-1024 time-averaging computer; for the A-60/C-1024 combination, the $(CH_3)_2CO$ peak provided the trigger signal.

Anticholinesterase activity was measured by a radiometric assay developed by Winteringham and Disney (1964). Bovine erythrocyte cholinesterase (Sigma Chemical Co.) was allowed to react with various levels of inhibitor for 60 minutes before the addition of either $1 \times 10^{-3}M$ or $5 \times 10^{-5}M$ acetylcholine. Aliquots were taken at 30, 60, and 90 seconds after substrate addition and the initial rate of reaction was calculated. This value was used to determine the I_{50} value.

Fly bioassay was performed on 1-day-old virgin nonresistant males. Carbaryl and the metabolite were dissolved in aqueous acetone (25%) at levels of 0.6, 1.2, and 1.8 mg. per ml. and were injected at 0.83 μl. per fly, corresponding to doses of 0.5, 1.0, and 1.5 μg. per fly. Mortality was recorded at 24 hours.

Carbaryl and the metabolite were plate-tested for microbial toxicity and mutagenicity according to the method described by Kolbye and Legator (1968). Six strains of histidine-requiring *Salmonella typhimurium* (G 46, C 117, C 340, C 527, C 120, and C 207) were tested for back mutation. These mutant strains are known to be reverted to a wild type by a number of mutagenic agents.

Gas-liquid chromatographic analysis was performed with a Packard Instrument Co. chromatograph under the following conditions: 6-foot × 4-mm. i.d. glass column containing 10% DC-200 on 80- to 100-mesh acid-washed Chromosorb W at 225° C.; inlet temperature, 225° C.; N_2, 60 ml. per minute; H_2, 30–35 ml. per minute; air, 300 ml. per minute; detection by flame ionization. Under these conditions, carbaryl chromatographed intact as a single component within 2 minutes of injection.

RESULTS AND DISCUSSION

The distribution of ^{14}C radioactivity in milk after the acute administration of ^{14}C-carbaryl to a lactating cow was previously reported (Baron, 1968; Baron *et al.*, 1968). Of the administered dose, 1% was recovered in the whole milk; approximately 0.13% of the administered dose was present as a chloroform-soluble fraction of skim milk. Thin-layer chromatography of this chloroform extract resulted in the separation of two metabolic components and a small quantity of material which remained at the origin. The largest component, which migrated with an R_f of 0.15 in ether-hexane (3 to 1), was found by radioassay to cochromatograph with a metabolite found in urine. This compound also cochromatographed with samples of a naphthyl-labeled carbaryl metabolite obtained from a rat liver microsome preparation and from cow's milk. [These samples were obtained through the courtesy of H. W. Dorough. The data concerning them were reported previously (Dorough, 1967).] As the quantity of material isolated from chloroform-soluble extracts of milk was not sufficient for characterization, the urinary components were used.

Results of urinary excretion of ^{14}C radioactivity following administration of carbaryl are shown in Figure 1. Approximately 30% of the ^{14}C dose was ultimately recovered in urine, more than half of that amount being excreted within 10 hours. Routine 24-hour samples of urine accounted for 95% of the material excreted in the urine as evaluated over a 74-hour test interval. Butanol, chloroform, and ethyl ether extractions were tested in an effort to remove the organic-soluble materials from urine. Liquid-liquid extraction for 24 hours with ethyl ether removed these materials with the least possible complication.

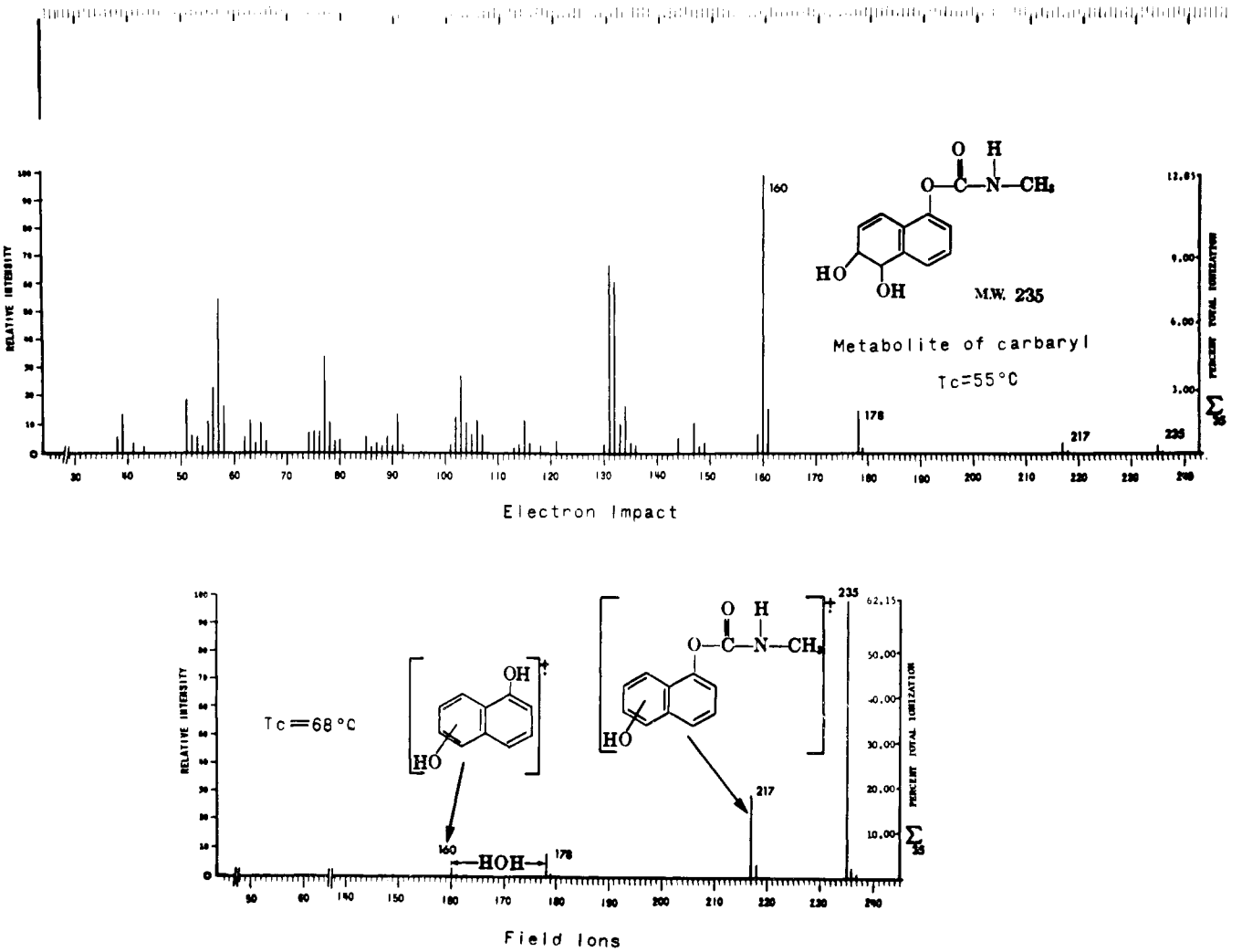

Figure 3. Mass spectrum of carbaryl metabolite

During the liquid extraction of urine, a crystalline material was observed in the water condenser. The material was removed; on analysis it was found to contain radioactivity and to have a sublimation (melting point) range of 134° to 139° C. (uncorrected). Although the melting point was high, infrared spectral analysis suggested that the material was ammonium bicarbonate, which probably had formed from the neturalization of $^{14}CO_2$.

Column chromatographic analysis of the ether extract showed the presence of four components (Figure 2). These compounds were collected and subjected to TLC separation. The smaller peaks (I and II) cochromatographed with 4-hydroxycarbaryl and 5-hydroxycarbaryl standards. Compound Ia, labeled as the *N*-methylol derivative of carbaryl, gave a positive test for hydroxymethyl urea following ammonolysis. An *N*-methylol carbaryl standard was not available for chromatographic confirmation. The largest metabolic component (III) migrated on TLC with an R_f identical to the metabolite found in milk (R_f 0.15) and was collected for further characterization. The compound collected from the Florisil column was further purified and resolved as a single component. The metabolite was crystallized from ether-hexane as small needles and had a melting point of 173–76° C. (corrected), gave no color test on the TLC plate with Gibbs' reagent or the diazonium fluoborate reagent, and was soluble in acetic acid, slightly soluble in acetone or ethanol, and insoluble in most other organic solvents.

The mass spectral data of the metabolite, establishing the molecular weight as 235, were again consistent with the structure of a methylcarbamate ester of dihydrodihydroxynaphthol. The high resolution mass spectral analysis confirmed the elemental composition of $C_{12}H_{13}NO_4$ (theoretical mass, 235.08445; actual mass, 235.08488). The fragmentation pattern obtained with an Atlas CH-4 spectrometer (Figure 3) was similar to the fragmentation pattern of aryl methylcarbamates obtained with a Time-of-Flight spectrometer (Damico and Benson, 1965). Differences in relative peak intensities may be due to differences in instrumentation. The molecular ion underwent hydrogen rearrangement to form the corresponding phenol (*m/e* 178) with the loss of methylisocyanate. The rearranged ions (*m/e* 178) then lost water to form the ion at *m/e* 160, which later lost CO. These processes were all confirmed with metastable peaks. A relatively intense peak was observed at *m/e* 217. This was not unexpected, because decomposition of the molecular ion to produce a completely aromatic system would be a favorable process. Thermal decomposition rather than electron impact was suspect because, in the electron impact mode, the ratio of the *m/e* 217 to *m/e* 235 peak was not consistent and a metastable peak was not observed. The field ionization data cannot establish whether the *m/e* 217 was due to a thermal decomposition or electron impact fragmentation. In an effort to resolve this anomaly in the spectral data, low voltage studies were employed. In these

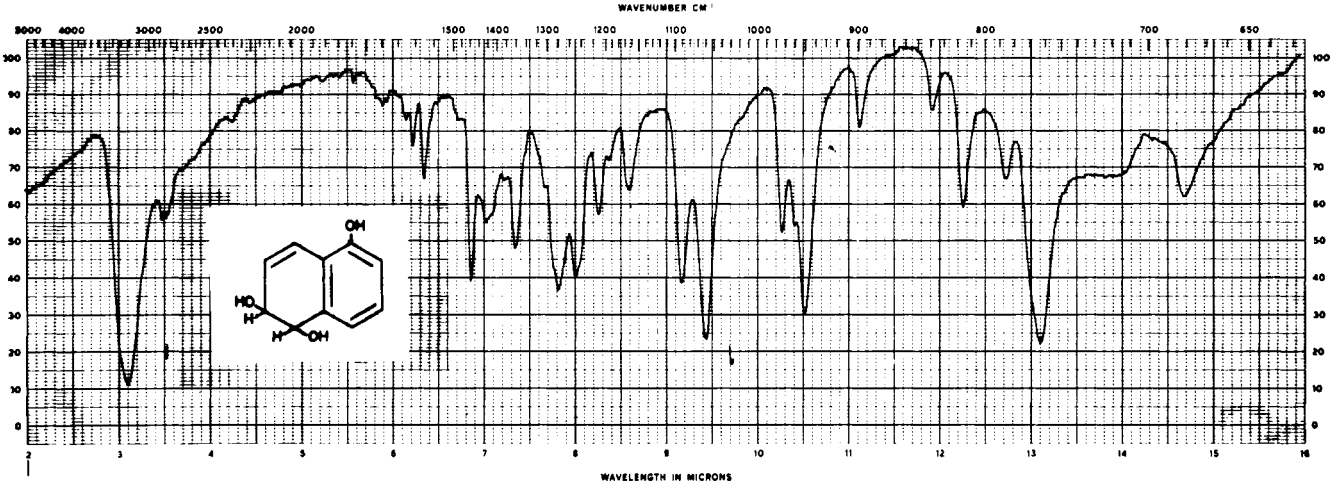

Figure 4. Infrared spectrum of 5,6-dihydrodihydroxy-1-naphthol obtained by alkaline hydrolysis of carbaryl metabolite

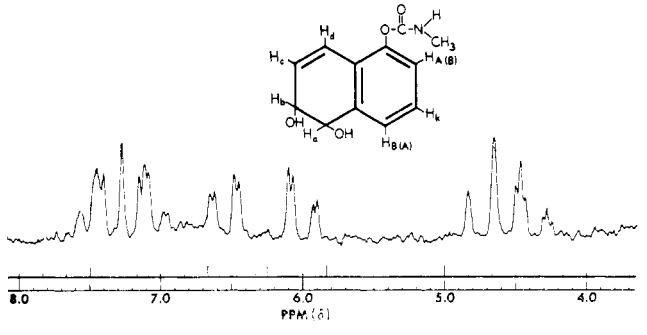

Figure 5. Partial NMR spectrum of carbaryl metabolite

Chemical shifts (δ, p.p.m.) for H_a: 4.70; for H_b: 4.40; for H_c: 6:00; for H_d: 6.50. Coupling constants (Hz): J_{ab} = 11; J_{ac} = $J_{ad} \approx 0$; av. of J_{bc} and J_{bd}: 2; J_{cd} = 10

studies, which indicated that thermal decomposition was occurring, the relative abundance of the *m/e* 217 ion increased with respect to the relative abundance of the molecular ion (*m/e* 235) at the low energies. However, low energy studies do not discount the possibility that some of the abundance of the *m/e* 217 may be due to an electron impact fragmentation pattern. Accordingly, the entire spectrum shown in Figure 3 may not be considered to be that of a pure compound, because some of the abundance of the *m/e* 160 ion is derived from *m/e* 217 as well as *m/e* 178. Unfortunately, this problem cannot be resolved, because, to obtain a mass spectrum, the sample must be heated to provide an adequate vapor pressure. In addition to these data, the mass spectra of 5-hydroxycarbaryl and 1,2-dihydro-1,2-dihydroxynaphthalene agreed with the mass spectrum of the metabolite, substantiating the mass analysis.

The ultraviolet spectrum of the metabolite was essentially the same as those of carbaryl and 1,2-dihydro-1,2-dihydroxynaphthalene. Two major peaks at 265 and 218 mμ were observed in all of the compounds examined.

Infrared analysis resulted in a spectrum identical to that published by Dorough and Casida (1964), and indicated the presence of an alpha unsaturated secondary alcohol, carbonyl function, conjugated double bond, NH, and either three or four free adjacent aromatic protons out of plane deformation. The spectrum is consistent with the postulated methylcarbamate ester of dihydrodihydroxy-1-naphthol. The infrared spectrum for the metabolite after alkaline hydrolysis (Figure 4) is consistent with a dihydrodihydroxy-1-naphthol structure. Elemental analytical data support the assigned formula for $C_{12}H_{13}NO_4$.

	C	H	N
Calculated	61.27	5.57	5.95
Found	61.19	5.24	6.14

Acidification of the metabolite on TLC with 1.0*N* HCl and development resulted in the separation of two materials, the original metabolite and a material which did not cochromatograph exactly with either 4- or 5-hydroxycarbaryl standards. Alkaline hydrolysis and acidification resulted in a phenolic compound which did not cochromatograph with 1,4- or 1,5-naphthalenediol. The diazonium fluoborate and Gibbs' reagent gave colored compounds with the acidified hydrolysis products. Infrared and ultraviolet spectral analysis of the compounds indicated that 5-hydroxycarbaryl and 1,5-naphthalenediol were formed after acid and alkaline treatment. The observation that the compound did not chromatograph exactly with the standard material may be evidence for the possible occurrence of a 6-hydroxycarbaryl or a 1,6-naphthalenediol contaminant which would not separate from the major components. Therefore, the metabolite, upon acidification, was probably converted to an aromatic system containing primarily the 5-hydroxyl groups with small amounts of a 6-hydroxyl compound.

The NMR spectrum of the metabolite (Figure 5) shows the bands listed in Table I and appears similar to that reported by Holtzman *et al.* (1967), which corresponds to the unsubstituted dihydrodihydroxynaphthalene. However, in contrast to their reports, fine structure was observed in both *AB* quartets. The chemical shifts of protons 1, 2, 3, and 4 could be assigned on the basis of the following well-established correlations (see Figure 5 for the labeling of protons).

(*a*) J_{ac} and J_{ad} for —CH_a—CH_b—CH_c=CH_d— are near zero.
(*b*) J_{ab} and J_{ac} for —CH_a—CH_b=CH_c— can be of the same order of magnitude.
(*c*) In Ar—CH_a=CH_b—, the shift of H_a relative to H_b is downfield.

The spectrum of the compound indicates the presence of one of the two possible isomers (cis or trans). In our opinion, the magnitude of J_{ab} (11 Hz) does not warrant the assignment of either isomer.

In addition, an examination of the 100-MHz spectrum shows that the band characteristic for the aromatic protons

consists of an *ABK* pattern, and that the spacing of lines in the *K* portion is compatible only with the presence of two *J*'s whose average is about 8 Hz. Thus, the proton *K* must be in the ortho position relative to the two others, and only the structure with CH_3NHCOO— in the peri position relative to H_1 can account for the NMR spectrum.

Gas chromatographic analysis of the metabolite using a flame ionization detector resulted in two peaks having retention times of 1.81 (85%) and 5.22 minutes (15%). The larger peak was collected and reinjected; a second peak was again present, indicating that the minor peak occurred as a result of decomposition. Both peaks were collected and separated by TLC; the same five components were present in each peak. These compounds cochromatographed identically with 1,5- and 1,6-naphthalenediol, 5- and 6-hydroxycarbaryl, and the unchanged metabolite. Considerably greater quantities of the carbamate esters were present in the early peak, indicating that decomposition was probably occurring at both the injection point and along the column.

Anticholinesterase activity of the metabolite indicated that carbaryl was approximately five times more active as an anticholinesterase agent than the metabolite. The I_{50} value for the metabolite was calculated as $5 \times 10^{-6}M$ and for carbaryl, $1.0 \times 10^{-6}M$. Studies at two different substrate levels (1×10^{-3} and $5 \times 10^{-5}M$) to evaluate the effect of substrate on anticholinesterase activity gave the same results. A limited number of fly bioassays were performed with carbaryl and the metabolite. The LD_{50} for carbaryl was approximately 1 μg. per fly. At the maximum concentration of metabolite tested (which approached the maximum solubility), no mortality was observed at the level of 1.5 μg. per fly.

Carbaryl and the metabolite, when examined for mutagenicity and microbial toxicity, exhibited no toxic or mutagenic effects under the conditions employed. However, this screening procedure offers limited information on the mutagenic properties of a compound.

Development of a quantitative analytical method for the determination of the 5,6-dihydro-5,6-dihydroxycarbaryl was attempted with Gibbs' reagent. After acid degradation and alkaline hydrolysis on the TLC plate, the reaction product gave a blue color. However, this could not be repeated in a test tube under a wide range of pH and reagent conditions. As the blue condensation reaction occurred only on TLC, the method of detection can at best be called semiquantitative.

Table I. Description of NMR Spectrum of Carbaryl Metabolite

Proton Type	Proton No.	Band Centers[a] (δ P.P.M.)	Description
Alicyclic	1 and 2	4.57 (4.65)	AB pattern; high-field part consists of two triplets
Olefinic	3 and 4	6.24 (6.19)	AB pattern; all lines are doublets
Aromatic	5,6 and 7	7.2 (7.3)	ABK pattern
$-NHCH_3$		2.83 (n.a.)	Doublet due to coupling with —NH-proton (J = 5 Hz)

[a] Numbers in parentheses correspond to chemical shifts reported by Holtzman *et al.* (1967).

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