# Studies of Carbamate Pesticide Metabolism Utilizing Plant and Mammalian Cells in Culture

Raymond K. Locke,* Vivian Beck Bastone,[1] and Ronald L. Baron[2]

One experimental and two commercial carbamate insecticides (carbaryl, Banol, and UC-34096), labeled with $^{14}C$ in selected positions, were introduced into the medium of human embryonic lung or tobacco cells in culture. Lung cells hydrolyzed Banol to the phenol, which was then conjugated as an *O*-glucuronide. UC-34096 was not metabolized by lung cells, but formed a spontaneous decomposition product. Tobacco cells in suspension culture incorporated 21% of the added carbaryl. A significant amount of the incorporated label was associated with cell debris following homogenization. The remaining label contained in the supernatant fraction consisted of an unidentified organoextractable metabolite, neutral conjugates of carbaryl, α-naphthol, and 5,6-dihydro-5,6-dihydroxycarbaryl, and acidic conjugates of carbaryl and α-naphthol.

Cell culture techniques have previously been used in studies of pesticide toxicity (Gabliks and Friedman, 1969; Litterest *et al.*, 1969) and, more recently, in studies of pesticide metabolism (Huang *et al.*, 1970). Previous studies from this laboratory on carbaryl metabolism by human embryonic lung cells in culture have shown that cell culture techniques are potentially useful for preliminary studies of pesticide degradation (Baron and Locke, 1970). The same techniques were used to study Banol (2-chloro-4,5-dimethylphenyl *N*-methylcarbamate) and UC-34096 [*N'*-(4-methylcarbamoyloxy-*o*-tolyl)-*N,N*-dimethylformamidine] metabolism. Carbaryl (1-naphthyl *N*-methylcarbamate) metabolism by tobacco cells in suspension culture was also investigated.

The metabolite that is produced from Banol in the lung cell system reflects a classical mammalian route of detoxification; it may represent a minor metabolite in the rat (Baron and Doherty, 1967). The water-soluble metabolites produced from carbaryl by tobacco cells in suspension culture possess altered organic moieties which have been previously reported in bean plant (Kuhr and Casida, 1967) and mammalian systems (Knaak *et al.*, 1965, 1968; Leeling and Casida, 1966). The nature of the conjugation systems involved in the metabolism of carbaryl by tobacco cells and the identity of an unconjugated organoextractable metabolite remain unknown.

## MATERIALS AND METHODS

**Thin-Layer Chromatography (tlc).** For the studies with either human embryonic lung cells or tobacco cells, 250-μ silica gel F-254 tlc plates were used. Plates were developed with one of the following solvent systems: A. ether–hexane (1:1, v/v); B. acetonitrile–hexane (9:1, v/v); C. methyl ethyl ketone–acetone–water (9.3:2.6:1.0, v/v/v); and D. isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v/v).

**Liquid Scintillation Counting.** All radioactivity measurements were made with a Packard TriCarb liquid scintillation spectrometer, Model 3365, using a scintillation phosphor previously described (Baron and Locke, 1970). Aqueous fractions were added directly to the phosphor; organic fractions were evaporated to dryness with air before the addition of phosphor. Counting efficiency was determined for each sample by the channels-ratio method.

**Human Embryonic Lung Cells in Monolayer Culture.** $^{14}C$-Radio-labeled Banol (Upjohn Co., Kalamazoo, Mich.) or UC-34096 (Union Carbide Corp., New York, N.Y.) was dissolved in either acetone or ethyl acetate and added to culture flasks. The solvent was allowed to evaporate; 15 ml of a culture medium previously described by Kelly and Darrow (1967) and $10^6$ cells of the L-132 strain of human embryonic lung (Flow Laboratories, Inc., Rockville, Md.) were then introduced. A monolayer culture developed at 37° C over a 3-day period, yielding 3 to 4 million cells. Controls, consisting of radio-labeled pesticide and medium but no cells, were prepared for each compound investigated.

Banol labeled with $^{14}C$ in the carbonyl, *N*-methyl, or ring-methyl position was added at a chemical concentration of 2 ppm and radiometric concentrations of $8.5 \times 10^{-2}$ μCi per ml for the carbonyl label, $6.7 \times 10^{-2}$ μCi per ml for the *N*-methyl label, and $6.3 \times 10^{-2}$ μCi per ml for the ring-methyl label.

UC-34096 labeled with $^{14}C$ in the ring-methyl position was added at a chemical concentration of 24 ppm and a radiometric concentration of $6.5 \times 10^{-2}$ μCi per ml.

The metabolism of carbaryl, labeled with $^{14}C$ in the ring-$C_1$ position, was reexamined in this cell line using methods and materials previously described (Baron and Locke, 1970).

After incubation, the cells were separated from the medium by centrifugation for 20 min at 7000 × *g* and washed three times with isotonic saline. The radioactivity retained in the cells was monitored by liquid scintillation counting.

The medium containing Banol metabolites was extracted with benzene by a procedure which had been shown to extract Banol quantitatively from the incubation medium. One volume of the medium was extracted with two volumes of benzene, the phases were separated, and the aqueous phase again extracted with one volume of benzene. The pooled benzene fractions were dried over $Na_2SO_4$ and aliquots of both phases were assayed for radioactivity. Aliquots of unextracted media were spotted on tlc plates and developed in solvent system D, and radioactive spots were compared with that of *N*-methylurea. Aliquots of the aqueous phase were treated with β-glucuronidase (Sigma Chemical Co., St. Louis, Mo., Bacterial-Type II) or with acid (0.1 *N* HCl,

Division of Pesticide Chemistry and Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C. 20204

[1] Hoffmann-La Roche Company, Nutley, New Jersey.

[2] Environmental Protection Agency, Washington, D.C.

30 min at 100° C) and then extracted with benzene. Aliquots from both phases were radioassayed and the organic extracts, containing the radio-labeled organic moieties of the water-soluble conjugates, were cochromatographed in solvent system C with known standards.

The medium containing UC-34096 was concentrated by high-vacuum rotary evaporation and applied directly to tlc plates. The plates were developed in solvent system B, examined by uv light, and scanned for radioactivity. Bands were eluted with acetone and analyzed by mass spectrometric and infrared spectrophotometric techniques. Details of the techniques were previously reported (Baron *et al.*, 1969).

**Tobacco Cells in Suspension Culture.** Callus of the XD strain of tobacco cells (kindly supplied by Philip Filner, MSU/AEC Plant Research Laboratory, Michigan State University) was transferred from agar to suspension culture. The XD strain, derived from the stem of *Nicotiana tabacum* L. var. Xanthi, grew well on M-1D, a chemically defined medium. The cell line and the composition of the culture medium have been previously described (Filner, 1965). The cell line was routinely subcultured by adding 30 ml of a 10-day, 130-ml suspension culture to 100 ml of fresh medium contained in 250-ml Erlenmeyer flasks. The stoppered flasks were incubated in the dark on a gyrorotatory shaker at 27° C. In order to maintain sterile conditions, the flasks were incubated in a closed room equipped with uv lighting.

$^{14}C_1$-Carbaryl was dissolved in acetone and added to sterile culture flasks. The flasks were stoppered and the solvent was allowed to evaporate overnight in a 37° C sterile chamber; 100 ml of medium and 30 ml of a 10-day suspension culture were then added and incubation was continued for 12 days. The chemical concentration of the labeled carbaryl in the incubation medium was 9.2 ppm, corresponding to a radiometric concentration of $1.2 \times 10^{-1}$ $\mu$Ci per ml. Controls were prepared by incubating radio-labeled carbaryl in cell-free culture medium. After 12 days, the cells were separated from the medium by centrifugation at 7000 $\times$ *g* for 20 min and the medium was filtered by vacuum. The cells were washed with three 75-ml portions of medium containing no radioactivity and then were frozen and lyophilized. No significant radioactivity could be detected in the final cell wash. Portions of the lyophilized cells were assayed for radioactivity by homogenizing them in phosphor and counting. Aliquots of the pooled cell washes and filtered medium were assayed for radioactivity and then lyophilized and stored frozen for later use.

The medium was extracted with methylene chloride by a procedure which quantitatively extracts carbaryl from the incubation medium (Baron and Locke, 1970). The organic extract was chromatographed on tlc plates in systems A and B and the radioactive spots were compared with those of known standards.

To investigate the nature of the radio-labeled metabolites of $^{14}C_1$-carbaryl incorporated into the tobacco cells, lyophilized cells were homogenized in distilled water and the homogenate was centrifuged for 20 min at 105,000 $\times$ *g*. The supernatant (containing the water-soluble metabolites) was decanted and assayed for radioactivity. The debris pellet was washed with water and centrifuged until no significant radioactivity appeared in the wash, and was then resuspended in water. Aliquots of the suspension were extracted with acetonitrile and methylene chloride or subjected to acid hydrolysis (0.1 *N* HCl or 1.0 *N* HCl, 30 min at 100° C) followed by organic extraction, and both phases were counted for radioactivity. The organic extracts of the treated suspensions were then chromatographed on tlc plates in solvent systems A and B along with known standards.

Portions of the supernatant from cell homogenization were subjected to either acid hydrolysis (0.1 *N* HCl, 30 min at 100° C) or to treatment with $\beta$-glucosidase (Sigma Chemical Co., Type II from almonds), with aryl sulfatase (Sigma Chemical Co., Type II from limpets), or with acid phosphatase (Sigma Chemical Co., Type II from potato). The treated aliquots were extracted with acetonitrile and methylene chloride and both aqueous and organic phases were assayed for radioactivity.

Another portion of the supernatant was chromatographed on a DEAE–cellulose ion-exchange column and the eluted $^{14}C$-radioactivity was monitored as previously reported (Baron and Locke, 1970). Two radioactive fractions were eluted and were subjected to enzyme treatments and acid hydrolysis followed by organic extraction and tlc of the organic extracts in systems A and B.

**Table I. Distribution of $^{14}C$-Radioactivity Added to Lung Cell Culture Media as Banol**

| Cell culture fraction | Recovered radioactivity, %: Carbonyl-Banol | Ring-methyl Banol | *N*-Methyl Banol |
|---|---|---|---|
| Washed cells | 0.003 | 0.005 | 3.8 |
| Benzene phase | 0.6 | 7.2 | 0.9 |
| Aqueous phase | 76.2 | 81.1 | 95.3 |
| $^{14}CO_2$ or loss | 23.2 | 11.7 | 0.0 |

## RESULTS AND DISCUSSION

**Human Embryonic Lung Cells in Monolayer Culture.** More than 90% of the $^{14}C$-Banol, whether labeled in the carbonyl, *N*-methyl, or ring-methyl position, was metabolized by lung cells to water-soluble conjugates within 3 days. The radio-label distribution is shown in Table I. Of the radioactivity added as Banol to control flasks containing cell-free culture medium, 100% was organoextractable after incubation.

The distribution of radioactivity in the media after removal of the cells, $\beta$-glucuronidase treatment, and benzene extraction of the treated media is shown in Table II. The data indicated that Banol had been hydrolyzed to the phenol and conjugated with glucuronic acid, since the radio-label was organoextractable after $\beta$-glucuronidase treatment of the medium containing ring-methyl-labeled Banol but not after treatment of the media containing *N*-methyl or carbonyl-labeled Banol. Tlc of the benzene extracts of all

**Table II. Distribution of $^{14}C$-Radioactivity in Benzene Extracts of Lung Cell Culture Media Containing $^{14}C$-Banol After Hydrolytic Treatments**

| Phase | Recovered radioactivity, %: Carbonyl-Banol | Ring-methyl Banol | *N*-Methyl Banol |
|---|---|---|---|
| | $\beta$-Glucuronidase treatment | | |
| Benzene | 0.7 | 90.8 | 1.8 |
| Aqueous | 99.3 | 9.2 | 98.2 |
| | Acid hydrolysis | | |
| Benzene | 8.8 | 24.0 | 8.0 |
| Aqueous | 80.9 | 76.0 | 92.0 |
| $^{14}CO_2$ or loss | 10.3 | 0.0 | 0.0 |

three β-glucuronidase-treated media, using System C and 2-chloro-4,5-dimethylphenol as a standard, revealed bands corresponding to the standard, as seen with uv light. Only the organic extract of the medium containing ring-methyl-labeled Banol had a radioactive spot at this same position. The extracts from β-glucuronidase-treated media containing *N*-methyl or carbonyl-labeled Banol exhibited a common radioactive spot 3 cm higher than that of the standard.

Table II also shows the distribution of radioactivity after acid hydrolysis and organic extraction of the media. Banol was stable to acid hydrolysis under the experimental conditions; the organic extract of the hydrolysate cochromatographed with authentic Banol and no radioactivity was lost. After tlc of the benzene extracts in System C, uv-quenching spots were observed with all three extracts at the position of 2-chloro-4,5-dimethylphenol, but a radioactive spot was observed at this position only in the extract of the medium containing ring-methyl-labeled Banol. The extracts from media containing Banol labeled in the *N*-methyl or carbonyl position showed a common radiopeak 2 cm lower than that of the standard.

It is postulated that a transcarbamylation product containing the $-CONHCH_3$ moiety of the parent carbamate is formed. No radioactive spot for *N*-methylurea was observed with aliquots from any of the culture media after tlc in System D was undertaken following treatment of the media by the method of Friedman and Lemin (1967), indicating that the intact carbamate group was not present in the water-soluble conjugates. The migration of radioactivity from all three media was identical with spots having an $R_f$ value of 0.48. From the β-glucuronidase data, it was ascertained that the single radioactive spot observed with medium containing ring-methyl-labeled Banol corresponded to the *O*-glucuronide of 2-chloro-4,5-dimethylphenol. The radioactive spots obtained from the media containing *N*-methyl or carbonyl-labeled Banol were at this same position in System D, indicating that a possible transcarbamylation product cochromatographed with a known glucuronide.

Collectively, these data indicated that Banol had been hydrolyzed by human embryonic lung cells to the phenol followed by conjugation with glucuronic acid as an *O*-glucuronide with concomitant production of a possible transcarbamylation product. The *N*-glucuronide of Banol has been proposed by Baron and Doherty (1967) as the major metabolite produced from Banol in the rat. The *O*-glucuronide produced from Banol by human lung cells may, however, represent the minor metabolite, Id, they reported. Transcarbamylation products arising from Banol metabolism by the rat were also reported in the same study.

$^{14}C$-Labeled UC-34096 was not metabolized by human lung cells, since at the end of the 3-day incubation period in media both with and without cells, 20% of the original radio-label was present as the parent compound and 80% was converted to 4-hydroxy-*N*-formyl-*o*-toluidine (Figure 1) as shown by mass spectrometric and infrared spectrophotometric data. No evidence was found for acidic conjugates by DEAE–cellulose column chromatography. The conversion of UC-34096 to its major hydrolytic product apparently was spontaneous and independent of the action of the lung cell.

Tlc in System B of a water solution of UC-34096 left standing for 40 days at room temperature yielded four compounds which, when characterized by mass spectrometry and infrared spectrophotometry, were believed to be: UC-34096 ($R_f$ 0.07), 4-(methylcarbamoyloxy)-*o*-toluidine ($R_f$ 0.38), 4-(methylcarbamoyloxy)-*N*-formyl-*o*-toluidine ($R_f$ 0.51), and 4-hydroxy-*N*-formyl-*o*-toluidine ($R_f$ 0.55) (Figure 1). Similar spontaneous decomposition behavior has been reported for another formamidine pesticide, Galecron (Knowles and Sen Gupta, 1969). These findings suggest that UC-34096 is not metabolized by the L-132 strain of human embryonic lung cells, but that the presence of calf serum additive and the incubation temperature caused extensive conversion of UC-34096 to the carbamate hydrolysis product, 4-hydroxy-*N*-formyl-*o*-toluidine, in preference to the other products found when UC-34096 decomposed spontaneously in water at room temperature.

Attempts to reproduce results obtained in a previous study of carbaryl metabolism by the L-132 strain of human embryonic lung cells (Baron and Locke, 1970) revealed that the extent of metabolism, as evidenced by the percentage of the added radioactivity present as water-soluble metabolites, varied from 11 to 87%. Studies aimed at detecting variations in the metabolism of carbaryl as a result of a freezing process used for storage of cells revealed no decrease in metabolism. The extent of carbaryl metabolism appeared to decrease with the number of transfers of the L-132 cell line. When a new culture was utilized, 46 and 87% of the parent compound were present as water-soluble conjugates in the first and second transfers, respectively. Metabolism thereafter decreased to 19% and remained at this level for three subsequent transfers.

Utilization of the L-132 strain of human embryonic lung cells in culture may prove useful in preliminary investigations of the metabolism of pesticides by mammals; however, attention must be given to reproducibility of results, in view of the many variables which may exert an effect upon pesticide metabolism in this system.

**Tobacco Cells in Suspension Culture.** Table III shows the radio-label distribution after a 12-day incubation of $^{14}C_1$-carbaryl with tobacco cells in suspension culture. Distributions independently obtained from replicate cultures agreed within 1%. The radioactivity present in the incubation medium was shown by cochromatography in Systems A and B to be unchanged carbaryl. Of the radio-labeled carbaryl incubated with cell-free medium as a control, 92% was recovered as unchanged carbaryl and 8% was recovered as α-naphthol.

The washed cells were homogenized in water and the resulting debris pellet was separated from the supernatant containing the water-soluble metabolites. The washed pellet was resuspended in water and radioassayed; 8.4% of the administered radioactivity was found in the cell debris, representing 40% of the radio-label incorporated into the cells.

When aliquots of the resuspended debris pellet were treated with acid and the hydrolysates subjected to acetonitrile and methylene chloride extractions, the amounts of

**Table III. Distribution of Recovered $^{14}C$-Radioactivity Added to Tobacco Cell Culture Medium as Carbaryl**

| Cell culture fraction | Recovered radioactivity, % |
|---|---|
| Medium | 65 |
| Cell wash | 14 |
| Washed cells | 21 (Total) |
| Washed cell debris | 8.4 |
| Neutral metabolites | 8.8 |
| Acidic metabolites | 3.8 |

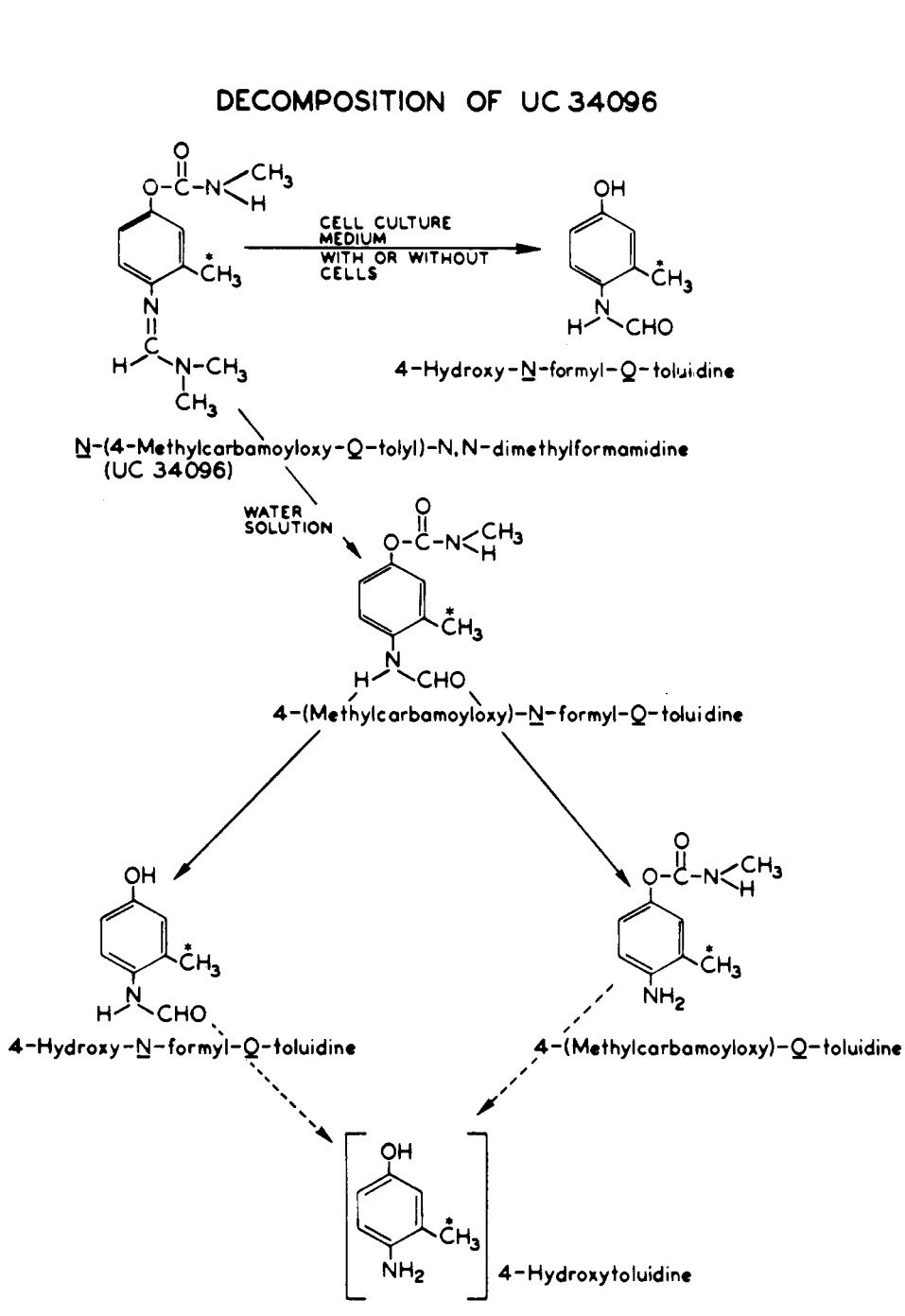


**Figure 1. Decomposition of UC-34096**

recovered radioactivity appearing in the organic phases were 13 and 11% for 1.0 *N* and 0.1 *N* HCl, respectively. Tlc of the organic extract of the 1.0 *N* HCl hydrolysate with known standards in System A revealed that 81.6% of the recovered $^{14}$C-radioactivity remained at the origin, 10.8% cochromatographed with carbaryl, and 7.6% cochromatographed with $\alpha$-naphthol. No 5,6-dihydro-5,6-dihydroxycarbaryl could be detected in the organic extract by tlc in System B with a known standard. $^{14}$*C*-Carbonyl-labeled carbaryl was stable to both hydrolytic conditions employed; 98% was recovered in the organic phases after extraction of the hydrolysates, indicating that little or no hydrolysis of the carbamate group had occurred. Thus, 40% of the radiolabel incorporated into the tobacco cells consisted of conjugates of carbaryl and of $\alpha$-naphthol tightly associated with the cell debris following cell homogenization. Whether actual conjugates were formed or there was simply intercalation within the cell wall lattice is not known.

The remaining 60% of the $^{14}$C-radioactivity incorporated into the cells (representing 12.6% of the administered label) was contained in the supernatant following cell homogenization. It could be separated into neutral (42%) and acidic (18%) fractions with DEAE-cellulose column chromatography and represented 8.8 and 3.8% of the added radioactivity, respectively. Of the radio-label in the cells, 42% was eluted with 0.01 *M* tris-HCl at pH 7.5 at the void volume of the column and was considered to represent neutral metabolites. No radio-label was eluted over two linear gradients which are known to elute sulfate and glucuronide conjugates (Knaak *et al.*, 1965). When 1.0 *M* tris-HCl (pH 7.5) was used to clear the column, 18% of the radioactivity present in the cells was eluted at the void volume and was considered to represent highly acidic conjugates. The fact that all of the $^{14}$C-radioactivity contained in the supernatant eluted in a single peak at the void volume with 0.1 *M* tris-HCl at pH 7.5, combined with the data obtained

with the linear gradients, indicated that the acidic metabolites eluted at a buffer concentration between 0.08 and 0.10 *M*.

No radioactivity over that of controls was noted in the organic phase when the supernatant, containing both the neutral and acidic metabolites, was treated with β-glucosidase, aryl sulfatase, or acid phosphatase and subsequently extracted. The water-soluble conjugates were therefore not *O*-glucosides, aryl sulfates, or phosphates.

The fraction from DEAE-cellulose column chromatography of the supernatant containing neutral metabolites was extracted using the acetonitrile and methylene chloride procedure and the organic extracts were chromatographed on tlc plates in Systems A and B with known standards. Of the radioactivity, 29% was organoextractable (representing 2.6% of the administered radioactivity) and contained a single unidentified unconjugated metabolite which migrated 2 cm lower ($R_f$ 0.54) in System B than 5,6-dihydro-5,6-dihydroxy-carbaryl ($R_f$ 0.67) and remained at the origin in System A. Standards of other possible metabolites failed to cochromatograph with the unidentified metabolite in System A or B. The water phase from this extraction was subjected to acid hydrolysis (0.1 *N* HCl, 30 min at 100° C) followed by organic extraction. The organic extract, containing 42% of the radioactivity, was cochromatographed with known standards in Systems A and B; 30% of the radio-label remained at the origin and 31% cochromatographed with 5,6-dihydro-5,6-dihydroxycarbaryl, 22% with α-naphthol, and 17% with carbaryl.

The nature of these neutral water-soluble conjugates is unknown. Although data obtained following β-glucosidase treatment would tend to eliminate the possibility of *O*-glucoside metabolites, there remains the possibility of a neutral *N*-glucoside with carbaryl or 5,6-dihydro-5,6-dihydroxy-carbaryl. Kuhr and Casida (1967) reported that the water-soluble metabolites produced from carbaryl by bean plants retained the carbamate moiety largely intact. *N*-Glucoside conjugates of carbaryl or its metabolites may be contained in metabolite fractions which these investigators found resistant to hydrolysis by β-glucosidase.

The acidic metabolite fraction was treated in the same manner as the fraction containing neutral metabolites. Radioassay of both the water and organic phases after organic extraction yielded only 3% of the recovered radio-label in the organic phase.

After treatment of the unextracted acidic fraction with acid phosphatase followed by organic extraction, no increase in radio-label was detected in the organic phase over that of the control, indicating the absence of acidic phosphate conjugates. Acid hydrolysis (0.1 *N* HCl, 30 min at 100° C) of the fraction containing acidic metabolites, followed by organic extraction and tlc of the organic extract (containing 30% of the recovered radioactivity) in Systems A and B with known standards, revealed that 28% of the radioactivity remained at the origin, 37% cochromatographed with carbaryl, and 35% cochromatographed with α-naphthol.

The nature of these acidic conjugates of carbaryl and α-naphthol is unknown. Data obtained following enzyme treatments would tend to eliminate phosphates and aryl sulfates as possible metabolites. A glucuronide conjugate would be extremely unlikely in this plant cell system, and both *O*- and *N*-glucuronides as well as sulfate conjugates would tend to be eliminated as possibilities since they were not eluted from DEAE–cellulose over gradients known to elute such conjugates (Knaak *et al.*, 1965). Possible amino acid or protein conjugates of carbaryl and α-naphthol would be consistent with the column chromatographic characteristics of the acidic metabolite fraction. Amino acid conjugation systems have been previously reported in plants (Kaslander *et al.*, 1962; Bach, 1961).

The use of tobacco cells in suspension culture may prove to be a useful tool in preliminary investigations of pesticide metabolism. The metabolism of carbaryl by these cells has been shown to be quantitatively reproducible. These cells are capable of oxidative metabolism, as is evidenced by the production of neutral conjugates of 5,6-dihydro-5,6-dihydroxycarbaryl from carbaryl. Hydrolytic and conjugation systems are present as shown by the production of both acidic and neutral conjugates of carbaryl and α-naphthol. The ability of this cell line to proliferate in a chemically defined medium makes its use in studies of metabolism more attractive than the use of plant or animal lines requiring media containing additives of indefinite composition and chemical effects.

NOTE ADDED IN PROOF. We have discovered that carbaryl and *N*-hydroxycarbaryl (1-naphthyl *N*-hydroxy, *N*-methylcarbamate) cochromatographed in Systems A and B under the conditions described in MATERIALS AND METHODS. When the metabolism of carbaryl by tobacco cells in suspension culture was reinvestigated, using a tlc solvent system capable of resolving *N*-hydroxycarbaryl from other common carbaryl metabolite aglycones, the metabolites present in both the tobacco cell supernatant and debris pellet fractions were those reported in the present study with one important exception: 100% of the *N* conjugates of carbaryl reported in the present study were characterized as *N–O* conjugates of *N*-hydroxycarbaryl by cochromatography of the aglycones in the newly applied solvents system. A communication describing the tlc characteristics of *N*-hydroxycarbaryl in solvent systems commonly used in carbaryl metabolism studies, as well as two tlc systems for the separation of the *N*-hydroxycarbaryl aglycone, is in preparation.

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