Utilization of Cell Culture Techniques in Carbaryl Metabolism Studies

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Although cell culture techniques have been used to examine the potential mutagenic properties of pesticides, they have been infrequently used for pesticide metabolism studies. Cell culture studies of metabolism have certain advantages over whole animal or plant studies especially in the isolation and purification of the metabolic products. In the present study cell culture techniques were used as a preliminary tool in order to examine the products of carbaryl (1-naphthyl N-methylcarbamate) degradation by cultures of the L-132 cell line derived from normal human embryonic lung (1). The metabolites produced were shown to be those which had previously been reported to occur in in vivo and in vitro studies of carbaryl degradation in plants, animals, and isolated systems (2, 3).

Materials and Methods

Carbaryl labeled with ^{14}C in the carbonyl position (Nuclear Chicago Corporation, Des Plaines, Illinois) was used at a chemical concentration of 10 ppm and a radiometric concentration of 1.43 μCi/ml; $^{14}C_1$-ring labeled carbaryl (synthesized) was used at concentrations of 10 ppm and 25.9 x 10^{-3} μCi/ml. The carbaryls were dissolved in either acetone or ethyl acetate and added to separate culture flasks, and the solvent was removed by evaporation. Fifteen ml of the culture medium (Eagle's Basal Medium with Earle's "BSS" and 10% calf serum; 2 mM in glutamine and containing 10,000 units each of penicillin and streptomycin per liter) was added and 10^6 cells of the L-132 strain of human embryonic lung cells were introduced. A monolayer culture was developed at 37°C over a 3-day period (4), yielding 3-4 million cells. The cells were separated from the medium by centrifugation for 20 min at 7000 x g and washed three times with isotonic saline, and the radioactivity was monitored with a Packard Tricarb scintillation counter (Model 3365). For all radioactivity measurements, the scintillation solution consisted of 500 ml toluene, 500 ml Cellosolve, 5.0 g 2,5-diphenyloxazole (PPO), 50.0 mg 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene (POPOP), and 33.3 g Cab-O-Sil®.

The medium containing metabolites was subjected to an acetonitrile-methylene chloride extraction procedure which we found to extract carbaryl quantitatively. To 1 volume of the medium, 2 volumes of acetonitrile were added. The pellet which formed was separated by centrifugation, washed with water, and assayed for radioactivity. Four volumes of methylene chloride were added to the supernatant, the mixture was shaken, and the fractions were separated. Two additional volumes of methylene chloride were used to further extract the aqueous fraction. The pooled methylene chloride fractions were dried over sodium sulfate and aliquots of both the aqueous and organic fractions were assayed for radioactivity. The organic

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fraction containing carbaryl was chromatographed by thin layer techniques and the radioactive spots were compared with those of known standards.

Aliquots of the water phase after the organic extraction were subjected to the following treatments: acid hydrolysis (0.1 N HCl, 30 min at 100°C); treatment with β-glucuronidase (Sigma Chemical Company, St. Louis, Mo., Bacterial-Type II); with aryl sulfatase (Sigma Chemical Company, St. Louis, Mo., Type II from Limpets); with acid or alkaline phosphatase; or with Pronase (Grade B, Calbiochem, Los Angeles, California)(5). The treated water phases were then extracted with an acetonitrile-methylene chloride mixture as above. Aliquots from both phases of the extractions were assayed for radioactivity and the organic fractions containing the organic moieties of the water-soluble conjugates were co-chromatographed and compared with known standards.

The following solvent systems were used for thin layer chromatography using plates coated with 250 μ Silica Gel GF-254:

I. Acetonitrile:hexane, 9:1 v/v.
II. Isopropanol:concentrated ammonia:water, 7:1:2 v/v.
III. Ether:hexane, 1:1 v/v.

System I was used to separate 5,6-dihydroxy-5,6-dihydrocarbaryl from several naphthalenediols, α-naphthol, carbaryl, and 4-hydroxy- and 5-hydroxycarbaryl. System II was used to establish the intactness of the carbamate moiety. System III was used to separate the naphthalenediols as well as to distinguish 4-hydroxy- and 5-hydroxycarbaryl from carbaryl.

Acidity of the water-soluble conjugates produced by cells in the medium containing 10 ppm ^{14}C-carbonyl-labeled carbaryl was established by ion exchange techniques. After the organic extraction of the culture medium, 0.25 ml of the water fraction was applied to 4.2 g of Rexyn 201 (Cl); the resin was then washed with 12 ml of water and 1.0 N HCl. Radioassay of the eluates showed that 89% of the ^{14}C-radioactivity was bound to the basic resin column and released with the acid wash. To chromatograph this acidic fraction, a column was prepared by packing 6 g of DEAE-cellulose (Cellex-D, Bio-Rad Laboratories, Richmond, California) batchwise in a 1.5 cm I.D. column, under flow conditions, to a final column height of 20.5 cm under ten pounds of pressure. One liter of 0.01 M Tris-HCl buffer (pH 7.6) was passed through the column before the sample application of one ml of the aqueous fraction (1.2×10^6 dpm ^{14}C-radioactivity). The metabolites were eluted using a linear gradient of 0.01 to 0.05 M Tris-HCl (pH 7.6), another linear gradient of 0.05 to 0.10 M Tris-HCl (pH 7.6), and finally 625 ml of 1.0 M Tris-HCl (pH 7.6). Three liters of 0.01 M Tris-HCl (pH 7.6) were passed through the column before application of a new sample. A constant flow-rate of 0.48 ml/min was maintained and the radioactivity was monitored with the aid of a flow cell (Packard Instrument Company). An authentic sample of 1-naphthyl-^{14}C-methylimidocarbonate-O-glucuronide, kindly supplied by Dr. J.B. Knaak, Mellon Institute, Pittsburgh, Pa., was also chromatographed under the same conditions.

Results and Discussion

^{14}C-carbaryl, introduced into the culture medium, was completely metabolized by L-132 cells within 3 days. The distribution of radioactivity is shown in Table 1.

TABLE 1

Distribution of Recovered ^{14}C-Radioactivity Added to Culture Medium As Carbaryl

Cell Culture Fraction	Carbonyl-Carbaryl, %	Ring-Carbaryl, %
Washed cells	0.02	0.1
Washed acetonitrile pellet	8.5	1.1
Organic phase of medium extraction	0.5	40.5
Water phase of medium extraction	46.8	25.6
$^{14}CO_2$ or loss	44.2	32.7

Medium containing ^{14}C-carbonyl-labeled carbaryl was extracted with acetonitrile-methylene chloride. The aqueous phase was subjected to acid hydrolysis, organic solvent extraction, and thin layer chromatography. The organic moieties of the water-soluble conjugates were characterized as 4-hydroxycarbaryl and 5,6-dihydroxy-5,6-dihydrocarbaryl. The 4-hydroxycarbaryl component accounted for approximately 92% of the radioactivity contained in water-soluble conjugates and the 5,6-dihydroxy-5,6-dihydrocarbaryl component accounted for 8%.

After β-glucuronidase treatment and organic solvent extraction of the culture medium containing either ^{14}C-carbonyl or $^{14}C_1$-ring-labeled carbaryl, the organic phase had no more radioactivity than that found in the medium with no enzyme, indicating that the water-soluble conjugates were not O-glucuronides. Similar treatment with aryl sulfatase, acid and alkaline phosphatase, and Pronase ruled out aryl sulfates, phosphates, and high molecular weight protein conjugates, respectively. These data suggested that the water-soluble conjugates were not a result of conjugation with α-naphthol which had been formed by hydrolysis of the carbamate group.

DEAE-cellulose column chromatography of the aqueous phase containing carbonyl-labeled carbaryl revealed a single radioactive peak which eluted at the position reported for 1-naphthyl methyl carbamate-N-glucuronide (3). An authentic sample of 1-naphthyl-^{14}C-methylimidocarbonate-O-glucuronide was also chromatographed and the results agreed well with those previously reported. N-glucuronides have been reported to be poor substrates for β-glucuronidase (5).

An effort was made to examine the metabolites through use of an ammonolysis reaction. The reaction of an intact carbamate and ammonia is expected to yield N-methylurea according to the following equation:

$$R\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}NHCH_3 + NH_3 \xrightarrow{H_2O} R\text{-}OH + NH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}NHCH_3$$

Twenty microliters of the water phases from an acetonitrile-methylene chloride extraction of medium containing either ^{14}C-carbonyl- or $^{14}C_1$-ring-labeled carbaryl were applied to a thin layer plate. These spots and a

spot of pure ^{14}C-carbonyl-labeled carbaryl were overspotted with 20 µl of System II prior to development. The plate was then allowed to incubate for 1 hour in a tank saturated with System II. After the plate was dried, a spot of α-naphthol was applied, and the plate was developed. The carbonyl-labeled standard gave a radiopeak with an R_f of 0.86 corresponding to that of N-methylurea (6). No radiopeak for N-methylurea was observed with aliquots from any of the culture media, indicating that the intact carbamate group was not present in the water-soluble conjugates. No $^{14}C_1$-naphthol was observed in the medium containing $^{14}C_1$-carbaryl.

Piperonyl butoxide was introduced (at a concentration of 50 ppm) into culture medium containing 10 ppm of carbonyl-labeled or ring-labeled carbaryl and the distribution of radioactivity was determined at the end of a 3-day incubation period. No differences could be observed in metabolites formed or their amounts as compared with those in the absence of piperonyl butoxide.

These data suggest that the L-132 strain of human embryonic lung cells in culture detoxify carbaryl by hydroxylation at the C_4 position and hydrolysis of the carbamate group, resulting in naphthalene-1,4-diol. The remainder of the compound is primarily conjugated as the N-glucuronide of 4-hydroxycarbaryl with a small fraction conjugated as the N-glucuronide of 5,6-dihydroxy-5,6-dihydrocarbaryl. The metabolites and their relative amounts remain unchanged with the addition of piperonyl butoxide to the culture medium.

The preliminary data in this investigation indicate that the L-132 strain of human embryonic lung cells in culture detoxifies carbaryl in the same manner that has been observed in both *in vivo* and *in vitro* studies using animals and *in vitro* enzyme systems. The use of cell cultures for preliminary investigation of metabolic conversions of pesticides therefore seems to be a potentially useful tool in studying the degradation of pesticides. However, the use of cell cultures from other human sources to provide evidence for detoxification of pesticides should be investigated further.

Summary

Radioactive carbaryl (1-naphthyl N-methylcarbamate), introduced into a culture of human embryonic lung cells (L-132 strain), was completely metabolized within 3 days to water-soluble conjugates or organoextractables in the medium. No radioactivity was found in the washed cells. Studies to elucidate the metabolic pathways of detoxification suggested that carbaryl was hydroxylated in the C_4 position of the ring and that approximately 40% of the labeled compound was hydrolyzed to form naphthalene-1, 4-diol. The remainder was found as the N-glucuronides of 4-hydroxycarbaryl and 5,6-dihydroxy-5,6-dihydrocarbaryl. The addition of piperonyl butoxide to the cell culture medium had no effect upon the detoxification of carbaryl.

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