CONCLUSION

The two photochemical reactions of heptachlor, photodechlorination and cage formation, take place *via* two different and distinct mechanisms. The formation of cage compound can be reversed at higher energy. This reverse reaction, or cage opening, proceeds through an excited state which is different from that of the forward reaction, or cage formation.

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Preforan Metabolism by Tobacco Cells in Suspension Culture

Raymond K. Locke* and Ronald L. Baron[1]

Preforan (*p*-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether), labeled with ^{14}C in the C_1 position of the 4-nitrophenyl moiety or in the CF_3 group, was introduced into the medium of tobacco cells in suspension culture. After 15 days of incubation, recovery of added radioactivity varied between 52 and 76%. Of the recovered radioactivity, 60 to 80% was incorporated into the cells, with the remainder appearing in the medium or cell wash. No unchanged parent compound could be detected in any fraction. Radiolabeled Preforan incubated in cell-free medium was recovered unchanged. Metabolites present in cells and culture medium produced from $^{14}C_1$-labeled Preforan were characterized as conjugates of 4-nitrophenol, including probable glucoside and amino acid or protein conjugates, together with unidentified acidic conjugates. $^{14}C_1$-labeled 4-nitrophenol appeared in the medium but not in the cells. Metabolites present in cells and medium produced from $^{14}CF_3$-labeled Preforan probably represent ^{14}C incorporation into natural products, resulting from oxidation and cleavage of the $^{14}CF_3$ group from the parent compound.

Tobacco cells in suspension culture have previously been utilized in studies of DNA replication in higher plant cells (Filner, 1965), in studies of enzyme synthesis, regulation, and activation (DeJong *et al.*, 1967, 1968; Filner, 1966; Filner and Varner, 1967), and in studies of amino acid metabolism (Olson, 1964). Kemp and Sutton (1971) investigated the rates of protein synthesis, accumulation, and degradation in callus cultures of tobacco cells. More recently, tobacco cells in suspension culture have been used in studies of carbaryl (1-naphthyl *N*-methylcarbamate) metabolism (Locke *et al.*, 1971). The same techniques were used to study the metabolism of Preforan [fluorodifen; C-6989; 4-nitrophenyl 2-nitro-4-(trifluoromethyl)phenyl ether; *p*-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether], a relatively new herbicide. Because residues of this herbicide may appear on crops destined for human consumption, a knowledge of the nature of the residue complex is essential.

The β-D-glucoside of 4-nitrophenol has been proposed as a major metabolite of Preforan in soybean and maize seedlings cultured in nutrient solution (Geissbühler *et al.*, 1969). Rogers (1971) reported that the metabolism of Preforan in soybean seedlings grown under similar conditions primarily involved a cleavage of the diphenyl ether linkage, resulting in degradation products yielding 4-nitrophenol upon acid hydrolysis. Cleavage of the diphenyl ether linkage of Preforan to yield unconjugated 4-nitrophenol has been reported in peanut seedlings, although the nature of the conjugated products produced was not investigated (Eastin, 1971).

The purpose of the present study was to determine the metabolites produced from Preforan by an established plant cell line, and to compare the *in vitro* metabolites produced with those reported in whole plant studies.

Plant cell culture techniques possess certain advantages over whole plant studies, especially with regard to the relative ease of growth, treatment, and isolation and purification of metabolites. A rapid *in vitro* system reflecting plant metabolism would greatly aid in indicating those pesticides whose residue complexes might pose a toxicological hazard to man.

MATERIALS AND METHODS

The XD cell line of *Nicotiana tabacum* L. var. Xanthi, the chemically defined M-1D medium, and the procedure used in subculturing have been previously described (Filner, 1965; Locke *et al.*, 1971). This cell line proliferates well in a chemically defined medium; therefore, in metabolism studies,

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it is preferable to plant cell lines requiring additives for growth (such as coconut milk) of unknown composition and chemical effects. In studies of carbaryl metabolism (Locke *et al.*, 1971) these cells have demonstrated enzyme systems capable of conjugation and of oxidative as well as hydrolytic transformations. Metabolism studies were conducted at Preforan concentrations of 1 or 2 ppm in the culture medium. These concentrations of nonradio-labeled Preforan had no observable effect upon cell growth; no differences were observed in the dry weights of cultures incubated for 15 days with Preforan, as compared with those of control cultures incubated in the absence of Preforan. To determine the possible effects of nonradio-labeled Preforan upon enzyme systems of the cells, acid phosphatase activity was determined in the supernatant fraction of cell homogenates, prepared after 15-day incubations of cells in culture medium with 1 or 2 ppm of Preforan, by using a modification of a blood serum method (Bergmeyer, 1963). The acid phosphatase activities of treated cultures were within the range established with several control cultures incubated in the absence of herbicide.

Preforan was labeled with ^{14}C in the C_1 position of the 4-nitrophenyl moiety or in the $-CF_3$ group (Ciba Agrochemical Company, Vero Beach, Florida). The labeled compounds were added to the culture medium at a chemical concentration of 1 or 2 ppm, corresponding to radiometric concentrations of 9.25×10^{-3} or 1.85×10^{-2} μCi per ml, respectively, for $^{14}C_1$-labeled Preforan and 9.31×10^{-3} or 1.86×10^{-2} μCi per ml for $^{14}CF_3$-labeled Preforan. Four flasks were prepared at each concentration for each radiotracer. Controls were prepared by incubating the radio-labeled compounds in cell-free culture medium.

The radioisotopes were dissolved in methylene chloride and added to sterile 250-ml Erlenmeyer flasks. The solvent was allowed to evaporate overnight in a 37°C sterile chamber; 100 ml of culture medium and 30 ml of a 10-day suspension culture of tobacco cells were then added with aseptic techniques and the flasks were incubated in the dark at 27°C on a gyrorotatory shaker for 15 days. In order to maintain sterile conditions the flasks were incubated in a closed room equipped with uv lighting.

After incubation the cells were separated from the medium by centrifugation at $7000 \times g$ for 20 min and the medium was filtered by vacuum. Aliquots of the filtered medium were taken for radioassay, and the medium was lyophilized and stored frozen for later use. The cells were washed with nonradioactive medium until no significant radioactivity could be detected in the wash; the pooled cell washes were then assayed for radioactivity. The cells were lyophilized and stored frozen for later use.

A Packard TriCarb liquid scintillation spectrometer (Model 3365) was utilized for all radioassays. A scintillation phosphor previously described (Baron and Locke, 1970) was utilized in the radioassay of particulate samples; thixotropic gel powder (Cab-O-Sil) was omitted from the phosphor when radioassaying nonparticulate samples. Aqueous fractions were added directly to the phosphor; organic fractions were evaporated to dryness before addition of phosphor. Effluents from chromatographic columns were radioassayed by adding aliquots of collected fractions directly to the phosphor. Highly radioactive standards eluted from chromatographic columns were radioassayed with the aid of a flow cell apparatus (Packard Instrument Company, Downers Grove, Illinois). Counting efficiency was determined by the channels-ratio method.

For thin-layer chromatography (tlc), precoated 250-μ Silica Gel F-254 plates (EM Reagents Division, Brinkmann Instruments, Inc., Westbury, N.Y.) or 250-μ plates prepared from Silica Gel G (Brinkmann Instruments) were used. The plates were developed with the following solvent systems: I. chloroform–ethanol–glacial acetic acid (90:5:5, v/v/v); II. chloroform–methanol–pyridine (20:1:2, v/v/v); III. 1-butanol–glacial acetic acid–water (4:1:1, v/v/v); IV. chloroform–methanol–17% (assay) ammonium hydroxide (2:2:1, v/v/v), lower phase; V. benzene–dioxane–glacial acetic acid (90:25:4, v/v/v). Radioactive spots were detected by radioassay of silica gel samples scraped from tlc plates, utilizing a scintillation phosphor previously described (Baron and Locke, 1970).

To investigate the nature of the radio-labeled metabolites of Preforan 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 fraction, containing the water-soluble metabolites, was decanted and assayed for radioactivity. The cell debris pellet was repeatedly washed with water and centrifuged until no significant radioactivity appeared in the wash. The cell washes were pooled for radioassay and the washed debris pellet was resuspended in water and assayed for radioactivity after homogenization in scintillation phosphor.

The supernatant fraction was extracted with methylene chloride by the following procedure, which has been shown to extract $^{14}C_1$- or $^{14}CF_3$-labeled Preforan quantitatively from the culture medium. Two volumes of methylene chloride were added to one volume of the supernatant fraction, the mixture was shaken, and the phases were separated. One additional volume of methylene chloride was used to further extract the water phase. The pooled methylene chloride extracts were dried over Na_2SO_4 and chromatographed by tlc techniques. Radioactive spots were compared to uv-quenching spots of known standards. The aqueous phase was hydrolyzed with acid (1 *N* or 4 *N* HCl; 30 min at 100°C) and extracted with methylene chloride, and the organic extract was chromatographed on tlc plates with known standards.

Portions of lyophilized cells were homogenized in distilled water, the homogenates were centrifuged for 20 min at $7000 \times g$, and the supernatant fractions (less than 3 ml in volume) were applied to a DEAE–cellulose ion-exchange column. Portions of lyophilized medium were reconstituted in distilled water for column chromatography. To prepare the column, 6 g of DEAE–cellulose (Cellex-D, Bio-Rad Laboratories, Richmond, Calif.) in 0.01 *M* tris-HCl buffer at pH 7.5 was packed batchwise in a 1.2-cm i.d. column to a final column height of 31.0 cm under 10 lb of pressure. A constant flow rate of 0.5 ml/min was maintained and 5-ml fractions were collected. At least 725 ml (16 void volumes) of 0.01 *M* tris-HCl buffer at pH 7.5 was used for elution; then an additional 150 ml of effluent was collected by using 1.0 *M* tris-HCl buffer at the same pH. At least 3 l. of 0.01 *M* buffer was passed through the column for equilibration before the application of a new sample.

Radioactive fractions from DEAE–cellulose column chromatography of the supernatant from tobacco cell homogenates or of culture medium were treated with acid (1 *N* or 4 *N* HCl; 30 or 90 min at 100°C), β-glucosidase (Sigma Chemical Company, Saint Louis, Mo.; emulsin from almonds), or aryl sulfatase (Sigma Chemical Company; from limpets, Type III). The treated fractions were then extracted with methylene chloride; both phases were radioassayed, and the organic extract was chromatographed on tlc plates.

Table I. Distribution of ^{14}C Radioactivity (%) Recovered after a 15-Day Incubation of Tobacco Cells in Culture Medium Containing $^{14}C_1$- or $^{14}CF_3$-Labeled Preforan[a]

Compound	Concentration, ppm	Recovery of added radioactivity	Cells	Medium	Cell wash
$^{14}C_1$-Preforan	1	70 ± 15	82 ± 5	10 ± 2	8 ± 3
$^{14}C_1$-Preforan	2	52 ± 7	78 ± 3	16 ± 2	7 ± 1
$^{14}CF_3$-Preforan	1	69 ± 10	60 ± 9	32 ± 8	9 ± 4
$^{14}CF_3$-Preforan	2	76 ± 14	60 ± 8	30 ± 5	10 ± 3

[a] Values are the arithmetic means of four identical simultaneous experiments per treatment group with the sample standard deviations.

For the enzyme treatments, 1 or 2 ml of the radioactive fractions were added to enough phosphate–citrate buffer at pH 5.2 (Dawson *et al.*, 1959) so that the total volume was 5 ml. A few drops of chloroform were added to each flask to retard bacterial growth, and the mixtures were incubated with 10 mg of β-glucosidase or 10 mg of aryl sulfatase for 1 hr at 37°C. To establish the activity of the aryl sulfatase and β-glucosidase enzyme preparations, *p*-nitrocatechol sulfate (dipotassium salt, Sigma Chemical Company) or *p*-nitrophenyl-β-D-glucoside (K&K Laboratories, Plainview, N.Y.) were tested as substrates under the same conditions; addition of 10 *N* NaOH following incubation produced a red or yellow color, respectively, indicating an active preparation. Little color development occurred in enzyme-free controls.

Radioactive fractions from column chromatography of cell supernatant or culture medium were concentrated by lyophilizing and reconstituting with a minimum of water. The concentrated fractions were chromatographed on tlc plates and radioactive spots were compared with uv-quenching or radioactive spots of known standards. Amino acids were detected by reaction with ninhydrin.

RESULTS AND DISCUSSION

Metabolism of $^{14}C_1$-Labeled Preforan. The distribution of recovered radioactivity shown in Table I was determined after tobacco cells had been incubated for 15 days in culture medium containing $^{14}C_1$-labeled Preforan at a chemical concentration of 1 or 2 ppm. There was little difference in the distributions of recovered radioactivity at the two chemical concentrations of Preforan used. Of the recovered radioactivity, approximately 78 to 82% was incorporated into the cells, 10 to 16% appeared in the culture medium, and 7 to 8% appeared in the cell wash. The recovery of added radioactivity varied from 52 to 70%. A loss of added radioactivity was also observed in controls. When $^{14}C_1$-labeled Preforan was added to cell-free culture medium, only radioactivity cochromatographing with authentic Preforan in tlc Systems I and V could be recovered from the medium after 1, 8, 11, or 28 days of incubation. Recovery of added radioactivity from controls declined linearly over this time period; 86% was recovered after 15 days of incubation. The loss of radioactivity from controls is probably due to precipitation of the radiotracer on the sides of the culture flask with evaporation of the culture medium. Some loss of radioactivity may represent evaporation of the compound itself. The additional loss of added radioactivity over that of controls observed in tobacco cell cultures may be due to loss of $^{14}CO_2$ as a result of metabolism or to incomplete assay of radioactivity contained in the cells. No solubilizing agent was employed in the radioassay of the cells; for this reason the values obtained may be somewhat low. Because of the oxygen requirement of the cells and consequent open nature of the culture system, no determination of $^{14}CO_2$ evolution was made. The culture medium and cells were examined for metabolites of $^{14}C_1$-labeled Preforan only in cultures incubated in medium containing Preforan at 2 ppm. In calculating the percentage of the total radioactivity recovered from all culture fractions represented by any specific metabolite found in the culture medium, the cell wash fraction has been considered diluted medium.

Table II. Distribution of Recovered ^{14}C Radioactivity (%) in Tobacco Cells after a 15-Day Incubation in Culture Medium Containing ^{14}C-Labeled Preforan

Fraction[a]	$^{14}C_1$ Label	$^{14}CF_3$ Label
Supernatant	92.0	79.0
Cell debris pellet	5.0	16.0
Pellet wash 1	3.0	4.0
Pellet wash 2	0.3	0.6
Pellet wash 3	0.1	0.4
Pellet wash 4	0.2	0.3

[a] Fractions obtained after cell homogenization and centrifugation at 105,000 × *g*.

The distribution of radioactivity contained in the tobacco cells is presented in Table II. Of the radioactivity contained in the cells (72% of the radioactivity recovered from all culture fractions), 92% was contained in the 105,000 × *g* supernatant fraction after cell homogenization. ^{14}C radioactivity contained in the washed cell debris pellet accounted for only 5% of the radio-label contained in the cells (4% of the radioactivity recovered from all culture fractions), and the pellet was therefore not further investigated.

The 105,000 × *g* supernatant was extracted with methylene chloride, and the organic extract (representing less than 1% of the radioactivity contained in the supernatant) was chromatographed on tlc plates in System I, along with known standards. Only one radioactive spot, which cochromatographed with 4-nitrophenol, was detected. The aqueous phase was hydrolyzed with acid (1 *N* or 4 *N* HCl, 30 min at 100°C) and extracted with methylene chloride, and the organic extract was chromatographed. ^{14}C-Preforan, labeled either in the C_1 position or in the $-CF_3$ group, was shown to be stable to acid hydrolysis under these conditions; all of the radioactivity added could be recovered in a methylene chloride extract after hydrolysis in 1 *N* or 4 *N* HCl, and tlc of the extract in System V revealed a single radioactive spot which cochromatographed with authentic Preforan.

The methylene chloride extract of the 1 *N* HCl hydrolysate of the aqueous phase contained 60% of the radioactivity, while 93% of the radioactivity contained in the 4 *N* HCl hydrolysate appeared in the organic phase. The extracts were spotted on tlc plates with known standards and developed in Systems I, II, and V. In each system only one radioactive spot, which cochromatographed with authentic 4-nitrophenol, could be detected. These data suggested that the water-soluble conjugates present in tobacco cells incubated

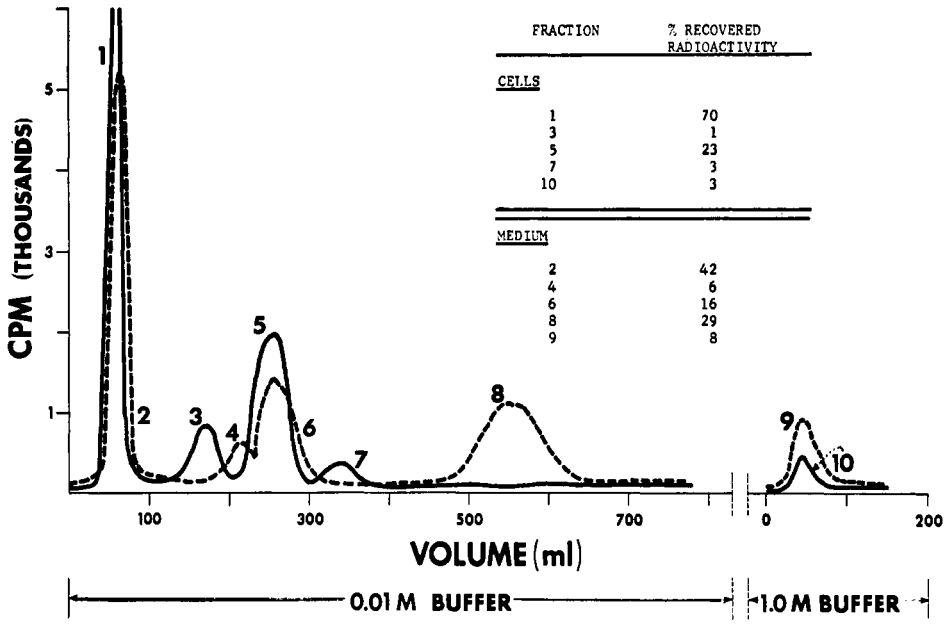

Figure 1. DEAE–cellulose column chromatography of supernatant (——) from tobacco cell homogenization and of culture medium (- - - - -) after 15 days of cell incubation in medium containing 2 ppm of $^{14}C_1$-labeled Preforan. Applied supernatant contained 550,000 dpm and medium contained 425,000 dpm of ^{14}C radioactivity. Tris-HCl at pH 7.5 was used for elution

in medium containing $^{14}C_1$-labeled Preforan were solely conjugates of 4-nitrophenol. These findings are essentially in agreement with results reported on Preforan metabolism by soybean and maize seedlings growing in nutrient solutions (Geissbühler *et al.*, 1969; Rogers, 1971). Although minor reduction of the nitro substituents of the parent compound were reported by these authors and by Eastin (1971), no 4-aminophenyl 2-amino-4-(trifluoromethyl)phenyl ether, 4-nitrophenyl 2-amino-4-(trifluoromethyl)phenyl ether, or 4-aminophenyl 2-nitro-4-(trifluoromethyl)phenyl ether arising from such reduction could be detected in the present study in any tobacco cell or culture medium fraction. No 4-aminophenol could be detected in acid or enzyme hydrolysates of water-soluble conjugates contained in any culture fraction.

When the culture medium was separated from cells following incubation and extracted with methylene chloride, 88% of the radioactivity remained in the aqueous phase. After tlc of the methylene chloride extract in System V, only one radioactive spot, which cochromatographed with 4-nitrophenol (R_f = 0.41), was found; no unchanged Preforan (R_f = 0.59) was detected. These data suggested that the ^{14}C radioactivity present in the culture medium represented water-soluble metabolites of Preforan, together with a small amount of unconjugated 4-nitrophenol.

In an effort to establish chromatographic similarities between metabolites present in tobacco cells and metabolites present in the culture medium, supernatant fractions from homogenization of lyophilized cells and reconstituted lyophilized culture medium were separately applied to a DEAE–cellulose ion-exchange column and eluted as previously described. The chromatographic profiles, shown in Figure 1, were essentially identical, with two exceptions: fraction 8 appeared in the medium but not in the supernatant, and fraction 7 appeared in the supernatant but not in the medium. Fraction 8 accounted for 29% of the radioactivity recovered from column chromatography of the culture medium, and represented 7% of the total radioactivity recovered from cells, culture medium, and cell wash after incubation. Fraction 7 accounted for 3% of the radioactivity that was recovered after column chromatography of the cell supernatant.

With the exception of fraction 8 (Figure 1), the radioactivity contained in the culture medium consisted of radio-labeled metabolites of Preforan originally contained in the cells, which had diffused throughout the medium with the rupture of a number of cell walls during the incubation process. Metabolite fractions present in the culture medium were assumed to be identical to those of the cell supernatant that eluted at a similar volume during column chromatography. This assumption was validated by cochromatography of pairs of these concentrated fractions in tlc systems. The absence of fraction 7 (Figure 1) from the culture medium may be explained by the great dilution of cell contents by the medium and the small percentage (3%) which fraction 7 represented of the total radioactivity contained in the cells.

When fraction 8 (Figure 1) was extracted with methylene chloride and both phases were assayed for radioactivity, 84% of the radioactivity was found in the organic phase. The organic extract was spotted on tlc plates with known standards and developed in Systems I and V. In either system only one radioactive spot could be detected and it cochromatographed with 4-nitrophenol. Therefore, 7% of the radioactivity recovered from all culture fractions following incubation was present in the medium as unconjugated 4-nitrophenol. A methylene chloride extract of the tobacco cell supernatant contained less than 1% of the radioactivity present in this fraction as radio-labeled 4-nitrophenol; the amount represented less than 0.8% of the radioactivity recovered from all culture fractions following incubation. These data suggested that $^{14}C_1$-labeled Preforan was metabolized by tobacco cells to ^{14}C-labeled conjugates of 4-nitrophenol, which were contained in the cells, and to unconjugated 4-nitrophenol, which was presumably excreted by the cells into the culture medium.

To investigate the nature of the metabolite(s) contained in fraction 1, which eluted at the void volume in the column chromatography of cell supernatant (Figure 1), aliquots were treated with β-glucosidsase, aryl sulfatase, or 4 *N* HCl. After β-glucosidase treatment, 77% of the radioactivity could be extracted into methylene chloride. Tlc of the organic extract in System I revealed a single radioactive spot, which cochromatographed with 4-nitrophenol. Only 2% of the radioactiv-

ity contained in the aryl sulfatase-treated aliquot could be extracted into methylene chloride. Of the radioactivity in fraction 1, 92% was labile to acid hydrolysis (4 *N* HCl, 30 min at 100°C); the methylene chloride extract of the hydrolysate contained only one radioactive spot which cochromatographed in System I with 4-nitrophenol. Aliquots of fraction 1, incubated with buffer in the absence of enzymes, contained no organoextractable radioactivity. These data suggested that fraction 1, representing approximately 55% of the radioactivity recovered from cells, medium, and cell wash, was a β-glucosidase-labile conjugate of 4-nitrophenol contained in the cells. This conjugate is probably the β-D-glucoside of 4-nitrophenol. This glucoside has been proposed as the major metabolite produced from $^{14}C_1$-labeled Preforan by soybean and maize seedlings (Geissbühler *et al.*, 1969).

Fraction 5 (Figure 1), representing 23% of the radioactivity recovered from column chromatography of tobacco cell supernatant, was treated with β-glucosidase, aryl sulfatase, or 4 *N* HCl. Only 7 to 8% of the radioactivity contained in the enzyme-treated fractions could be extracted into methylene chloride. No radioactivity could be extracted into methylene chloride from aliquots of fraction 5 incubated with buffer in the absence of enzymes. After hydrolysis in 4 *N* HCl (30 min at 100°C), 94% of the radioactivity of fraction 5 was organoextractable; the organic extract of the hydrolysate contained a single radioactive spot which cochromatographed in System I with 4-nitrophenol. These data suggested that fraction 5, present in the cells and representing 18% of the radioactivity recovered from all culture fractions following incubation, was a water-soluble conjugate of 4-nitrophenol other than an aryl sulfate or *O*-glucoside.

Fraction 5 (Figure 1) eluted from DEAE–cellulose with an elution volume at which three ^{14}C-radio-labeled amino acid standards were observed to elute. Fraction 5 eluted at 240 ml; L-glutamate, L-leucine, and L-aspartate eluted at 250, 225, and 235 ml, respectively, under the same experimental conditions. Therefore fraction 5 may represent an amino acid or protein conjugate(s) of 4-nitrophenol contained in the cells. Amino acid conjugation systems have been reported previously in plants (Andreae and Good, 1957; Bach, 1961; Bach and Fellig, 1961; Kaslander *et al.*, 1962; Klämbt, 1961; Massini, 1959).

Minor acidic metabolites of $^{14}C_1$-labeled Preforan contained in the cell supernatant (represented by fractions 3, 7, and 10) were not further investigated.

Metabolism of $^{14}CF_3$-Labeled Preforan. Table I shows the distribution of radioactivity after a 15-day incubation of tobacco cells in culture medium containing $^{14}CF_3$-labeled Preforan at a chemical concentration of 1 or 2 ppm. The distribution of radioactivity was essentially the same for the two chemical concentrations of Preforan used. Of the recovered radioactivity, 60% was incorporated into the cells, 30 to 32% appeared in the medium, and 9 to 10% appeared in the cell wash. The recovery of added radioactivity varied from 69 to 76%. $^{14}CF_3$-labeled Preforan incubated in cell-free culture medium was recovered unchanged following incubation. The loss of added activity from controls containing $^{14}CF_3$-labeled Preforan paralleled that of controls containing $^{14}C_1$-labeled Preforan. Culture medium and cells were examined for metabolites of $^{14}CF_3$-labeled Preforan only in cultures incubated in medium containing Preforan at 2 ppm. In calculating the percentage of the total recovered radioactivity present in any fraction, the cell wash has been considered diluted culture medium.

The distribution of radioactivity contained in the cells is presented in Table II. Of the radioactivity contained in the cells (47% of the radioactivity recovered from all culture fractions), 79% was contained in the 105,000 × *g* supernatant fraction after cell homogenization. The washed cell debris pellet accounted for 16% of the radio-label contained in the cells (10% of the radioactivity recovered from all culture fractions). The pellet was not further investigated.

A methylene chloride extract of the 105,000 × *g* supernatant fraction contained only 0.6% of the radioactivity present in the supernatant, and was not further investigated. The aqueous phase was hydrolyzed with acid (1 *N* or 4 *N* HCl; 30 min at 100°C), and the hydrolysate was extracted with methylene chloride. The methylene chloride extracts contained 8.4 or 8.9% of the radioactivity present in the 1 *N* or 4 *N* HCl hydrolysates, respectively, and were not further investigated. The water-soluble products present in the cell supernatant were probably natural products, stable to acid hydrolysis, as opposed to labile conjugates containing moieties of the parent herbicide. Such ^{14}C-incorporation products have been reported previously in metabolism studies of $^{14}CF_3$-labeled trifluralin and benefin in plants (Kearney and Kaufman, 1969).

In an effort to establish chromatographic similarities between metabolities present in tobacco cells and metabolites present in the culture medium, supernatant from homogenization of lyophilized cells and reconstituted lyophilized culture medium were separately applied to a DEAE–cellulose ion-exchange column and eluted as previously described. The chromatographic profiles thus obtained are shown in Figure 2.

Although their elution volumes from DEAE–cellulose were not identical with column chromatography of tobacco cell supernatant and culture medium, the following pairs of radioactive fractions were presumed to represent the same metabolite(s): 11 and 12, 14 and 15, 16 and 17, and 19 and 20 (Figure 2). The percentage that each fraction represented of the total radioactivity recovered from column chromatography of either cell supernatant or culture medium tended to support the assignments made. With these assignments the chromatographic profiles of cell supernatant and culture medium were essentially identical, with two exceptions: fraction 13 appeared in the cell supernatant, but not in the medium, and fraction 18 appeared in the medium, but not in the cell supernatant. The fact that fraction 18, appearing in the culture medium only, was chromatographically different from fraction 17 (contained in the cell supernatant) and fraction 16 (contained in the culture medium) was established by tlc of the concentrated fractions in System III. As expected, fractions 16 and 17 cochromatographed, while fraction 18 exhibited a distinctly different chromatographic behavior.

The absence of fraction 13 (Figure 2) from the culture medium may be explained by the great dilution of cell contents by the medium and the small percentage (1%) the fraction represented of the total radioactivity contained in the cells.

Fraction 18 (Figure 2) was thought to represent unconjugated 2-nitro-4-(trifluoromethyl)phenol, which would result from cleavage of the ether linkage of Preforan, together with 4-nitrophenol. No fraction 18 could be detected in the cells, and its presence in the culture medium suggested that it was excreted by the cells into the medium. When fraction 18 was extracted with methylene chloride or ethyl acetate, the radioactivity in the organic extract was only 1 or 11%, respectively. Concentrated unextracted fraction 18 failed to cochromatograph with authentic 2-nitro-4-(trifluoromethyl)-phenol in System V. This fraction, representing 7% of the radioactivity recovered from all culture fractions after incu-

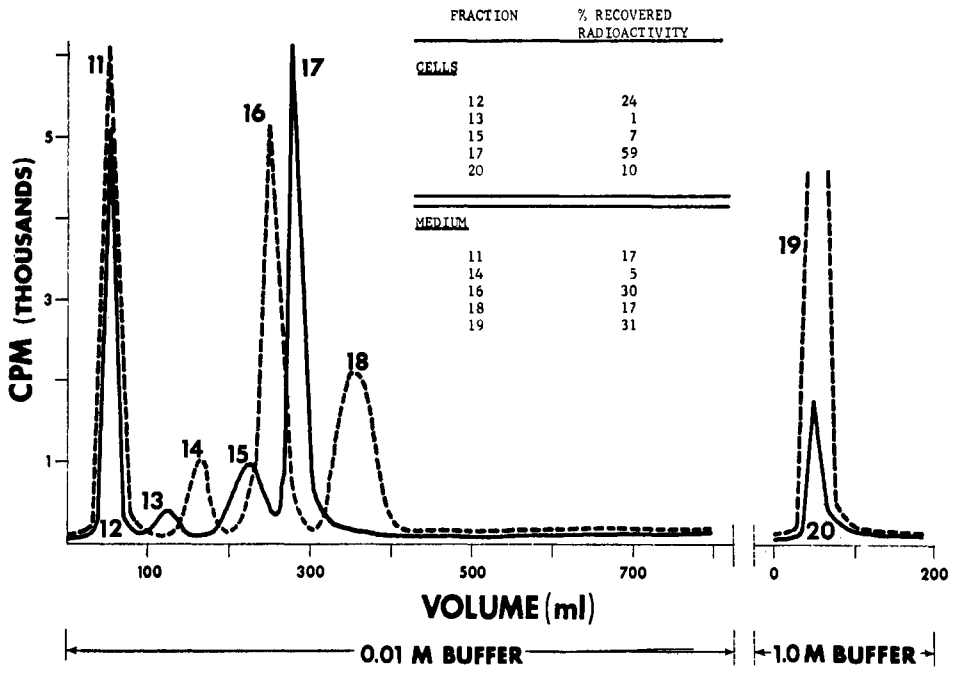

Figure 2. DEAE-cellulose column chromatography of supernatant (——) from tobacco cell homogenization and of culture medium (- - - -) after 15 days of cell incubation in medium containing 2 ppm of $^{14}CF_3$-labeled Preforan. Applied supernatant contained 447,000 dpm and medium contained 610,000 dpm of ^{14}C radioactivity. Tris-HCl at pH 7.5 was used for elution

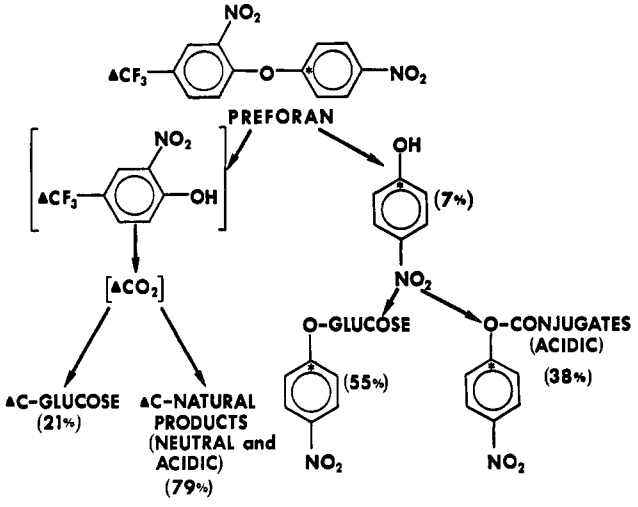

Figure 3. Proposed scheme for the metabolism of Preforan by tobacco cells in culture. Numbers in parentheses represent percentages of ^{14}C radioactivity recovered from cells, culture medium, and cell wash after incubation of cells with $^{14}C_1$-labeled (*) or $^{14}CF_3$-labeled (▲) Preforan. Compounds in brackets were not isolated. ^{14}C-radio-labeled 4-nitrophenol and one acidic natural product fraction were found only in culture medium with $^{14}C_1$-labeled or $^{14}CF_3$-labeled Preforan, respectively

bation, therefore represented a metabolite(s) other than the suspected phenol.

Aliquots of concentrated fractions 18 and 16 (Figure 2) were spotted with standards of 21 L-amino acids on tlc plates and developed in Systems III and IV. A major portion of the radioactivity contained in both fractions cochromatographed in these systems with a group of amino acids: tyrosine, leucine, phenylalanine, and tryptophan. Aliquots of concentrated fractions 18 and 16 were then applied to another tlc plate, and the plate was incubated for 15 min in ammonia vapor and dried. Fresh aliquots were applied and the plate was developed in System V. No additional radioactive spots were detected in the aliquots subjected to ammonolysis. No radioactivity could be detected in either fraction at the position of 2-nitro-4-(trifluoromethyl)phenol. The major portion of the radioactivity of both fractions was present at the origin, as was a phenylalanine standard. These data suggested that fraction 18 and 16 (Figure 2) each contained at least two radiolabeled components, one of which cochromatographed in each case with a group of amino acids in several solvent systems. These fractions were not further investigated. Fraction 16, from the medium, and fraction 17, from the cell supernatant, jointly accounted for 47% of the radioactivity recovered from all culture fractions following incubation.

An aliquot of concentrated fraction 11 (Figure 2), which eluted at the void volume in the column chromatography of culture medium, was hydrolyzed with acid (1 *N* HCl; 30 min at 100°C). The pH of the hydrolysate was adjusted to 7.5 with 6 *N* KOH. The hydrolysate and an aliquot of nonhydrolyzed concentrated fraction 11 (pH 7.5) were chromatographed in System III with a ^{14}C-labeled D-glucose standard. No additional radioactive spots could be detected in the hydrolysate, as compared with nonhydrolyzed fraction 11. Of the radioactivity contained in nonhydrolyzed concentrated fraction 11, 43% cochromatographed with D-glucose (R_f = 0.23), with the remainder at the origin. With hydrolyzed concentrated fraction 11, 15% of the radioactivity cochromatographed with D-glucose, with the remainder at the origin. This suggested acid degradation of the sugar. Fraction 11 (medium) and fraction 12 (cell supernatant) jointly accounted for 21% of the radioactivity recovered from all culture fractions following incubation. These fractions probably represent ^{14}C-labeled glucose, as opposed to a glucoside conjugate, based on the chromatographic behavior of hydrolyzed and nonhydrolyzed samples. After 5 months of storage at 4°C and pH 7.5, nonhydrolyzed concentrated fraction 11 failed to cochromatograph with D-glucose in System III. As op-

posed to the chromatogram obtained immediately following reconstitution of lyophilized fraction 11, the chromatogram of stored reconstituted fraction 11 contained a single radioactive spot (R_f = 0.59), far removed from a D-glucose standard (R_f = 0.24). These data suggested degradation of the sugar with storage at a slightly alkaline pH.

Since the metabolites of $^{14}CF_3$-labeled Preforan, both in the cells and culture medium, appeared to represent ^{14}C incorporation into natural products, fractions 13, 14, 15, 19, and 20 were not further investigated. ^{14}C incorporation into natural products has been reported in studies of $^{14}CF_3$-labeled trifluralin and benefin metabolism in plants (Kearney and Kaufman, 1969). Further studies of the fate of the 2-nitro-4-(trifluoromethyl)phenyl moiety of the Preforan molecule will necessitate the use of Preforan ring-labeled with ^{14}C in that moiety.

A simplified scheme for the metabolism of Preforan by tobacco cells in culture is presented in Figure 3. The metabolites produced from $^{14}C_1$- and $^{14}CF_3$-labeled Preforan by this rapid *in vitro* system are in good agreement with metabolites reported in whole plant studies (Geissbühler *et al.*, 1969; Kearney and Kaufman, 1969; Rogers, 1971). For this reason, the use of tobacco cells in suspension culture may prove a useful tool in studies of pesticide metabolism by plant tissue.

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Isopropyl Carbanilate (Propham) Metabolism in the Chicken: Balance Studies and Isolation and Identification of Excreted Metabolites

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Leghorn hens were given a single oral dose of either isopropyl-(2-^{14}C) carbanilate (A) or isopropyl carbanilate-[phenyl-^{14}C(U)] (B). Total carbon-14 excreted in the urine accounted for 79.8% of the activity given as A and 87.0% of the activity given as B during the 48-hr collection period; the fecal elimination accounted for 6.4 and 7.0% of carbon-14 given as A and B, respectively. When A was given, 6.7% of the carbon-14 was expired, whereas only trace amounts of expiratory carbon-14 were detected when B was given. Carbon-14 remaining in the hens 48 hr after dosing accounted for 1.2 and 1.5% of the activity given as B and A, respectively. Eggs collected for 12 days after dosing contained 0.075% of the carbon-14 given as a single oral dose of B. Urinary metabolites were identified as the glucuronide conjugate of isopropyl 4-hydroxycarbanilate (I), *p*-aminophenyl sulfate, the sulfate ester of isopropyl 3-methoxy-4-hydroxycarbanilate, the sulfate ester of isopropyl 4-hydroxycarbanilate (VI), the 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate (VII), an incompletely identified conjugated form of isopropyl 3,4-dihydroxycarbanilate, and the sulfate ester of isopropyl 3-hydroxycarbanilate. The feces contained I, VI, VII, and isopropyl 4-hydroxycarbanilate.

Isopropyl carbanilate (propham) is used extensively as a selective preemergent and postemergent herbicide. The effect of this compound on the growth of plants (Templeman and Sexton, 1945) and its effect at the cellular level in plants (Ennis, 1948) and animals (Timson, 1970) have been reported. The toxicology of propham in several animals has been investigated (FAO/WHO, 1964). More recently, Holder and Ryan (1968) reported that rats given unlabeled propham intraperitoneally excreted the sulfate ester of isopropyl 4-hydroxycarbanilate in the urine.

Since propham is widely used as a pesticide it seems likely that chickens and other farm animals may become exposed to this compound either directly or indirectly through feedstuffs.

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