Metabolism of Carbofuran in Alfalfa and Bean Plants

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The metabolism of benzofuranyl-7a-C^{14} and carbonyl-C^{14} carbofuran were investigated in alfalfa and bean plants. Carbofuran was administered to the plants via soil application. The major metabolites in alfalfa were identified as the glycosides of 3-hydroxycarbofuran (37.3%), 2,3-dihydro-3,7-dihydroxy-2,2-dimethylbenzofuran (18.5%), and 2,3-dihydro-7-hydroxy-2,2-dimethyl-3-oxobenzofuran (20%). Identical products were found in the bean plant. Mono and oligosaccharide residues were found for each aglycone by silica gel chromatography. The acetylated monoglycoside of 3-hydroxycarbofuran gas chromatographed on a 6 ft 5% SE-30 column as a single component.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) may be used effectively in soil or in foliar applications to control the alfalfa weevil. A major factor in the consideration of this insecticide for use on alfalfa is the nature of the residue which may remain in or on the crop. The metabolism of carbofuran in the intact cotton plant and in isolated cotton and corn leaves was studied by Metcalf *et al.* (1968). Dorough (1968) reported on the fate of carbofuran in the intact bean plant.

This study was undertaken to supply information on the nature of the metabolites in alfalfa and bean plants arising from soil application of carbofuran. The alfalfa hays containing carbofuran metabolites were used in subsequent studies involving the dairy cow and rat.

METHODS

Chemicals. Benzofuranyl-7a-C^{14} (0.145 mCi per mmole) and carbonyl-C^{14} (2 7 mCi per mmole) labeled carbofuran (Figure 1) were synthesized, respectively, by J. F. Start, FMC Corp., Princeton, N.J., and J. W. Woods, Mallinckrodt/Nuclear, St. Louis, Mo. Ring and carbonyl labeled 3-hydroxycarbofuran (2,3-dihydro-3-hydroxy-2,2-dimethyl-7-benzofuranyl methylcarbamate) were synthesized by S. E. Forman, FMC Corp., Princeton, N. J., according to the procedures of Metcalf *et al.* (1968) using labeled carbofuran. Ring and carbonyl labeled 3-oxocarbofuran (2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranyl methylcarbamate) were obtained as byproducts during the reaction. The ring labeled phenols, 2,3-dihydro-7-hydroxy-2,2-dimethylbenzofuran (7-phenol), 2,3-dihydro-7-hydroxy-2,2-dimethyl-3-oxobenzofuran (3-keto-7-phenol), and 2,3-dihydro-3,7-dihydroxy-2,2-dimethylbenzofuran (3,7-diol) were obtained by sodium hydroxide hydrolysis of the corresponding carbamates. The nonlabeled carbamates and phenols were synthesized by S. T. Young, Niagara Chemical Division, FMC Corp., Middleport, N.Y., using carbofuran as a starting product as previously described for the labeled compounds.

Treatment of Alfalfa and Bean Plants. Two to three year old alfalfa plants (Narragansett variety) were transplanted from field plots to 12 in. × 12 in. tar paper pots (Mennes Nursery, Tonawanda, N.Y.). After a 30-day recovery period the plants were cut back to 3 in. in height and were individually treated with 18.0 mg of ring-C^{14} or 9.0 mg of carbonyl-C^{14} labeled carbofuran. In practice, carbofuran was dissolved in 25 ml of a 1 to 1 acetone-water solution and applied to the soil through five sand-filled holes at the base of the plant. Twenty plants were treated with carbofuran-carbonyl-C^{14} and 50 plants were treated with carbofuran-ring-C^{14} and placed in a greenhouse. Thirty days after carbofuran application the plants were cut, pooled according to label, and dried in a forced air oven at 60° C.

Two week old bean plants (pinto) possessing two trifoliate leaves were treated individually in 3 in. × 3 in. pots with 5.0 mg of ring-C^{14} and carbonyl-C^{14} labeled carbofuran and 3-hydroxy carbofuran. In practice, carbofuran was dissolved in 10 ml of a 1 to 1 acetone-water solution and applied to the soil through three sand-filled holes at the base of the plant. The plants were held for a period of 1 week in a plant growth chamber, cut off at the soil level and dried overnight at 60° C in a forced air oven.

Analysis of Metabolites. Representative alfalfa leaf and stem samples were ground in a Model ED-5 Thomas-Wiley mill equipped with a 2.0 mm sieve. Total C^{14} metabolites (ring and carbonyl labels) were determined by bomb combustion and scintillation counting of 0.5 g samples (Knaak *et al.*, 1965). Metabolites were removed from alfalfa (5.0 g) by extracting first with diethyl ether and then with methanol. Extractions were carried out in a Soxhlet extractor over a 24 hr period. The individual extracts were assayed for C^{14} (0.1 to 1.0 ml aliquots) by direct liquid scintillation counting.

The bean plants were ground in a mortar and pestle and extracted with methanol. Carbon-14 analysis was made by direct liquid scintillation counting. All extractions were performed on an individual basis according to label (ring-C^{14} and/or carbonyl-C^{14}).

Silica Gel Chromatography. The diethyl ether and methanol extracts from alfalfa and the methanol extracts from the bean plants administered carbofuran and 3-hydroxy carbofuran were individually concentrated to 10 ml by distillation. One ml aliquots (ring-C^{14} or carbonyl-C^{14} labels) were absorbed onto 3 to 4 g of silica gel (Davison Chemical Division, W. R. Grace and Co., Baltimore, Md., Grade 923) and allowed to air dry. The gel containing the metabolites was then slurried in hexane and added to the top of a 1.5 × 24 cm column of silica gel previously packed in hexane. The metabolites were eluted using a series of stepwise gradients of hexane to ethyl acetate, ethyl acetate to acetonitrile, and acetonitrile to methanol. The gradients were formed by decreasing the percentage of each starting solvent by 10% (*i.e.*, 100, 90, 80) until a concentration of 10% was reached. In actual practice, each step consisted of 50 ml of solvent. Ring labeled carbofuran, 3-hydroxycarbofuran, 3-oxocarbofuran, the 7-phenol, the 3-keto-7-phenol and the 3,7-diol were used to characterize by chromatography the phenols and carbamates.

The conjugated phenols and carbamates, glycosides G, H,

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Table I. Silica Gel Chromatographic Analysis of the Metabolites of Carbofuran Extracted from Alfalfa with Diethyl Ether

Metabolites	Metabolites Expressed as % of Total C^{14} Recovered from Column I[a]	II[b]
A. 2,3-dihydro-7-hydroxy-2,2 dimethylbenzofuran	30.0	0.0
B. 2,3-dihydro-7-hydroxy-2,2 dimethyl-3-oxobenzofuran	6.8	0.0
C. 2,3-dihydro-3,7-dihydroxy-2,2 dimethylbenzofuran	13.9	0.0
D. carbofuran	41.8	20.0
E. 3-hydroxycarbofuran	7.5	80.0

[a] I. carbofuran-ring-C^{14}. [b] II. carbofuran-carbonyl-C^{14}. Diethyl ether extracted 3.0% of the ring-C^{14} metabolites and 5.0% of the carbonyl-C^{14} metabolites from dried alfalfa. Small quantities of the glycosides of these metabolites were extracted with diethyl ether. Ninety percent of the C^{14} placed on the column was recovered.

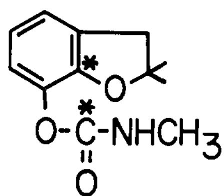

Figure 1. Structural formula of carbofuran and position of C^{14}

I, and J as eluted from the silica gel column (methanol extracts from alfalfa) were individually concentrated to 5.0 ml by reduced pressure distillation. The glycosides and residual solvent were dissolved in 100 ml of 0.1*N* HCl and refluxed for 1.0 hr. The acid hydrolyzate was cooled, neutralized to pH 7.0 using sodium hydroxide, and extracted with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and reduced in volume to 1.0 to 2.0 ml by distillation. The agylcones were absorbed on to 3 to 4 g of silica gel, air dried, and column chromatographed on silica gel using only the hexane to ethyl acetate stepwise gradient.

Gel Filtration. The individual glycosides (alfalfa) of 3-hydroxycarbofuran-carbonyl-C^{14} (H, I, and J) previously separated on silica gel and dissolved in 5.0 ml of distilled water were placed on a 1.5 × 150 cm column of Bio-Gel P-2 (100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif.) packed in water. The glycosides were eluted from the column with water at 100 ml per hr. The flow rate was controlled by a Dialgrad pump (Instrument Specialties Co., Lincoln, Neb.). Ten ml fractions were collected and 1.0 ml aliquots were analyzed for C^{14} by scintillation counting.

Gas Chromatography. The alfalfa glycosides of 3-hydroxycarbofuran-carbonyl-C^{14} (G, H, I, and J) from the silica gel column were individually dissolved in 10 ml of acetic anhydride after the removal of solvent by distillation. One to two drops of methanesulfonic acid were added and the mixture heated to 100° C for 1 hr. The reaction mixture was cooled, poured into ice water, and neutralized to pH 7.0 using 5*N* sodium hydroxide. The acetylated products were extracted with chloroform and concentrated by distillation to 1.0 ml. Ten microliters of this solution was gas chromatographed. In addition, the ring-C^{14} glycosides H from alfalfa as well as the carbonyl-C^{14} glycoside H from the bean plant were prepared for gas chromatography as previously described for the carbonyl-C^{14} glycosides.

Gas chromatography was carried out on a Barber-Coleman 5000 gas chromatograph equipped with a radioactive monitor

Table II. Silica Gel Chromatographic Analysis of the Metabolites[a] of Carbofuran-C^{14} in Alfalfa

Metabolites	Metabolites Expressed as % of Total C^{14} Recovered from Column I[b]	II[c]
A. 2,3-dihydro-7-hydroxy-2,2-dimethylbenzofuran	2.6	0.0
B. 2,3-dihydro-7-hydroxy-2,2-dimethyl-3-oxobenzofuran	2.5	0.0
C. 2,3-dihydro-3,7-dihydroxy-2,2-dimethylbenzofuran	2.7	0.0
D. carbofuran	5.2	3.0
E. 3-oxocarbofuran	0.0	0.0
F. 3-hydroxycarbofuran	9.6	7.0
G. glycoside(s) } H. glycoside(s)	35.0	57.5
I. glycoside(s)	37.2	26.4
J. glycoside(s)	5.2	6.1

[a] Methanol extracts from dried alfalfa (70 to 72% of the total C^{14} metabolites). [b] I. carbofuran-ring-C^{14}. [c] II. carbofuran-carbonyl-C^{14}. Ninety percent of the C^{14} placed on the column was recovered.

(RAM) and hydrogen flame detector (10 to 1 split). Six foot × 5 mm i.d. glass columns packed with 5% SE-30 on Applied Science Gas-Chrom Q (80 to 100 mesh) were used. The column was programmed at 3° C per min with an injection port temperature of 300° C and a detector temperature of 340° C. Helium (75 ml per min) was used as a carrier gas.

Enzyme Studies. Carbofuran-carbonyl-C^{14} metabolites obtained by methanol extraction from alfalfa and separated on a silica gel column were combined and incubated with 50 mg of Takamine Cellulase 4000 (Miles Laboratories Inc., Chemicals Division, Elkhart, Ind.) in 15.0 ml of 0.05*M* sodium acetate buffer, pH 5.25 for 1 hr after the solvent was removed by distillation. The reaction mixture was neutralized to pH 7.0 and freeze-dried on Chromosorb W (30 to 60 mesh) using the procedure of Knaak *et al.* (1967) for dilute aqueous solutions of metabolites. The products were eluted from the Chromosorb W with methanol, concentrated by distillation to 1.0 ml, and absorbed onto 3 to 4 g of silica gel. The methanol was evaporated off and the sample was applied as a slurry in hexane to the top of a 1.5 × 24 cm silica gel column and chromatographed as previously described for the methanol extracts.

In another study, glycoside(s) I, isolated from the carbofuran-ring-C^{14} and 3-hydroxy carbofuran-ring-C^{14}-treated bean plants were individually incubated with cellulase as previously described for the residues. The products were individually freeze-dried and eluted off Chromosorb W with methanol. The methanol was removed by distillation and the products were acetylated with 10 ml of acetic anhydride and two drops of methanesulfonic acid and gas chromatographed as described in the section on gas chromatography.

RESULTS

Carbofuran applied to the soil of potted alfalfa plants at 9.0 mg per pot for the carbonyl-C^{14} label and 18.0 mg per pot for the ring-C^{14} label resulted in C^{14} residues in the stems and leaves amounting to 76 ppm and 306 ppm of carbofuran, respectively, on a dry matter basis. Diethyl ether extracted 3.0% of the carbofuran-ring-C^{14} labeled metabolites from the dried alfalfa and 5.0% of the carbonyl-C^{14} labeled metabolites. Seventy to 72% of the total ring and carbonyl labeled metabolites were extracted by methanol.

The ether and methanol extracts were examined individually by silica gel chromatography. The metabolites and their percentages are given in Tables I and II for diethyl ether and

Table III. Silica Gel Chromatographic Analysis of the Aglycones Liberated by Acid Hydrolysis of the Glycoside[a] Obtained from Carbofuran-Ring-C^{14}

Aglycones	Aglycones Expressed as % of the Total[b] C^{14} Recovered from Column[c] G	H	I
A. 2,3-dihydro-7-hydroxy-2,2-dimethylbenzofuran	0.5	0.12	0.5
B. 2,3-dihydro-7-hydroxy-2,2 dimethyl-3-oxobenzofuran	6.8	1.8	10.2
C. 2,3-dihydro-3,7-dihydroxy-2,2-dimethylbenzofuran	5.0	2.3	10.0
D. 3-hydroxycarbofuran	12.7	5.8	16.5

[a] Glycosides were obtained from the chromatogram in Figure 2A.
[b] Glycoside G, 25%; H, 10%; I, 37.2%. [c] Figure 2A.

methanol extracts. The methanol extracts chromatographed as shown in Figure 2A. Table III gives the results obtained on a silica gel column with the aglycones derived from the ring labeled glycosides G, H, and I by acid hydrolysis. More than one aglycone was present in each peak. The carbonyl-C^{14} labeled glycosides G, H, I and J (Figure 2A) contained only 3-hydroxy carbofuran.

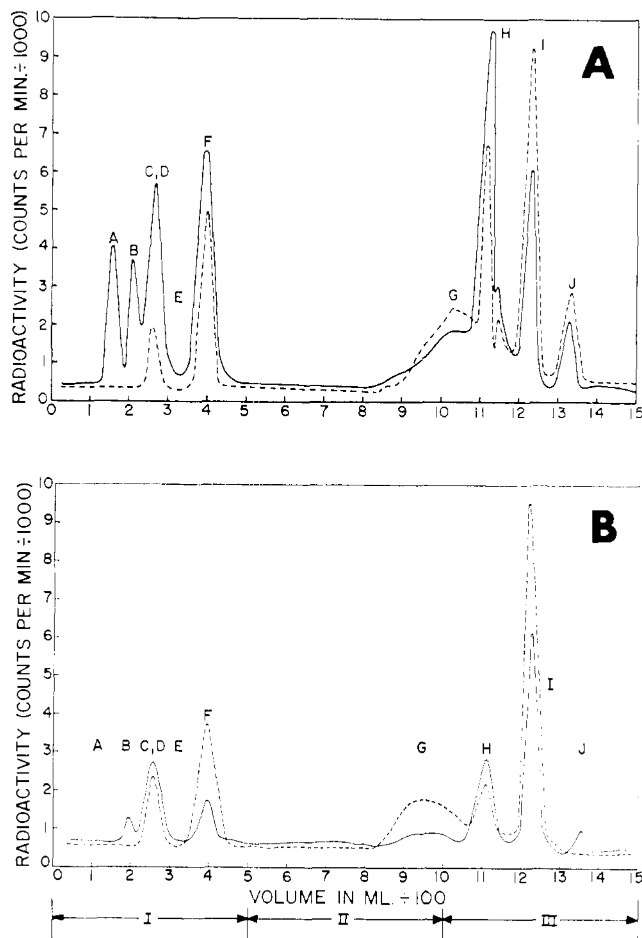

Figure 2. Silica gel chromatogram of the carbofuran metabolites

A. Alfalfa, 30 days after soil application
B. Bean, 7 days after soil application
—— Ring-C^{14} label
- - - - Carbonyl-C^{14} label
Elution program: I. Hexane to ethyl acetate; II. Ethyl acetate to acetonitrile; III. Acetonitrile to methanol; A 1.5 × 24 cm column of silica gel was used

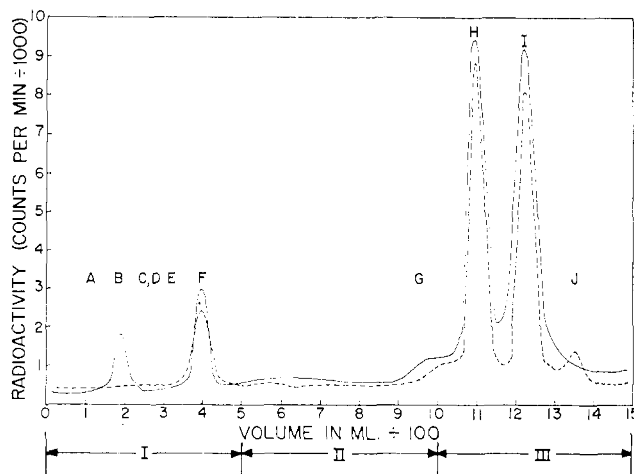

Figure 3. Silica gel chromatogram of the metabolites found in bean plants 7 days after soil application of 3-hydroxycarbofuran

—— Ring-C^{14} label
- - - - Carbonyl-C^{14} label
Elution program: I. Hexane to ethyl acetate; II. Ethyl acetate to acetonitrile; III. Acetonitrile to methanol; A 1.5 × 24 cm column of silica gel was used

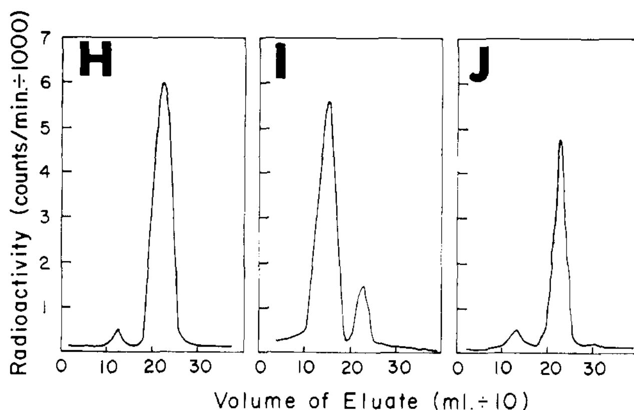

Figure 4. Chromatogram of the individual glycosides of 3-hydroxycarbofuran-carbonyl-C^{14} (H, I, and J) from alfalfa on a 1.5 × 150 cm column of Bio-Gel P-2 using water as the eluting solvent. The glycosides were previously separated on a silica gel column. See Figure 2

The ring-C^{14} and carbonyl-C^{14} labeled carbofuran metabolites from the bean plant chromatographed on a silica gel column as shown in Figure 2B in a matter similar to the alfalfa metabolites of carbofuran in Figure 2A. The major metabolites were the glycosides (G, H, and I). 3-Hydroxycarbofuran in the bean plant exhibited a similar pattern in the glycoside region to that of carbofuran in alfalfa and in the bean plant, as shown in Figure 3.

The individual glycosides of 3-hydroxycarbofuran-carbonyl-C^{14} (H, I, and J) eluted from the Bio-Gel P-2 column (Figure 4) as symmetrical peaks. On Bio-Gel P-2, oligosaccharides are eluted in order of decreasing size (Raftery *et al.*, 1969). In this study glycoside I eluted prior to glycoside H, while glycoside J eluted in the same position as H.

The acetylated carbonyl-C^{14} glycosides of 3-hydroxycarbofuran (G, H, I, and J) obtained from alfalfa were individually gas chromatographed on a 6 ft, 5% SE-30 column. Figure 5 gives the results for glycoside H. 3-Hydroxy carbofuran was liberated during the acetylation reaction. Glycosides G, I, and J did not gas chromatograph after acetylation.

Table IV. Silica Gel Chromatographic Analysis of Carbofuran-Carbonyl-C^{14} Alfalfa Metabolites[a] before and after Incubation with Takamine Cellulase 4000

Metabolites	Metabolites Expressed as % of Total C^{14} Recovered from Column	
	I[b]	II[c]
D. carbofuran	3.0	3.7
F. 3-hydroxycarbofuran	7.0	25.6
G. glycoside	31.7	32.4
H. glycoside	21.6	22.9
I. glycoside	26.4	4.4
J. glycoside	10.3	11.0

[a] Methanol extracts from dried alfalfa. [b] I. carbonyl-C^{14} labeled metabolites before incubation. [c] II. carbonyl-C^{14} labeled metabolites after incubation. For conditions see text.

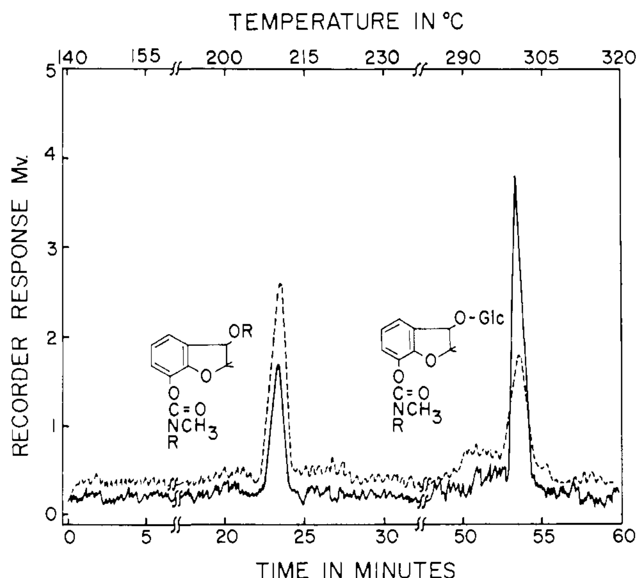

Figure 5. Gas chromatogram of an acetylated glycoside of 3-hydroxycarbofuran (H, Figure 2A)

——— Carbonyl-C^{14}
- - - - Ring-C^{14}

Glc, acetylated monosaccharide; R=$CH_3\overset{O}{\overset{\|}{C}}$; Inst., Barber Coleman 5000, radioactive monitor; Column, 6 ft × 5-mm i.d.; Support, Gas Chrom Q, 80/100 mesh; Liquid phase 5% SE-30; Flow rate, 75 ml per min.; Carrier gas, helium; Injection port temp, 300°C

3-Hydroxy carbofuran appeared as a product of the acetylation reaction in the case of glycosides I and J. The results for the ring-C^{14} labeled glycoside(s) H are given in Figure 5. The major products were 3-hydroxy carbofuran and its glycoside. The chromatogram shows that other ring-C^{14} labeled glycosides are present. The carbonyl-C^{14} glycosides, Figure 2B, from the bean plant were examined in a similar manner. The glycoside of 3-hydroxy carbofuran (H) in the bean plant was chromatographically identical to the corresponding glycoside (H) in alfalfa. Glycosides G, I, and J from the bean plant did not gas chromatograph after acetylation.

Table IV gives the hydrolytic results obtained with Takamine Cellulase 4000 on the carbofuran-carbonyl-C^{14} glycosides. The only noticeable change in composition was a decrease in the percentage of glycoside I and an increase in the percentage of free 3-hydroxy carbofuran. No evidence was obtained for the conversion of glycoside I to H or glycoside J to glycosides, I, H, or to free 3-hydroxycarbofuran. In the gas chromatographic studies, glycoside(s) I, obtained

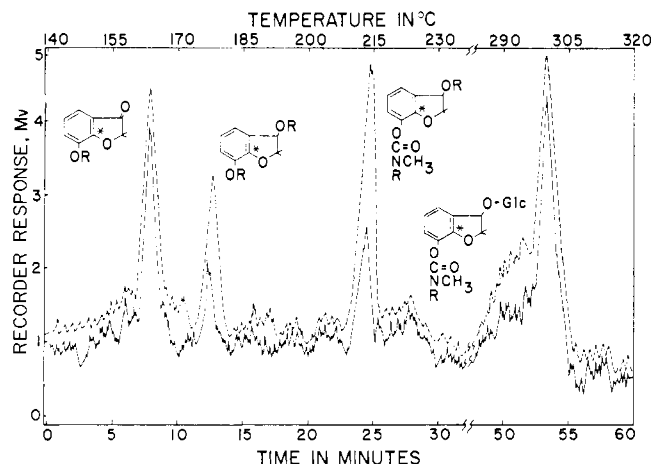

Figure 6. Gas chromatogram of the ring-C^{14} products of glycoside I obtained by cellulase treatment and acetylation

——— Glycoside I was obtained from bean plants administered carbofuran-ring-C^{14}. See Figure 2B
- - - - Glycoside I was obtained from bean plants administered 3-hydroxycarbofuran-ring-C^{14}. See Figure 3

Glc, acetylated monosaccharide; R = $CH_3\overset{O}{\overset{\|}{C}}$; See Figure 4 for conditions

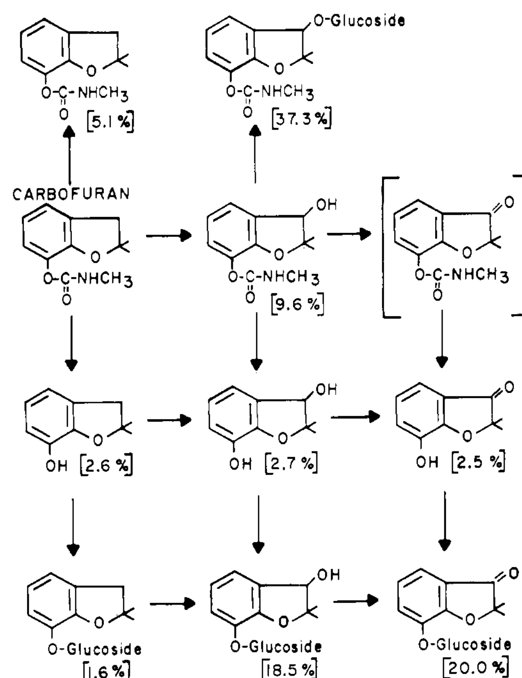

Figure 7. Metabolic pathway of carbofuran in alfalfa

Metabolites and their percentages were determined 30 days after soil application

from bean plants administered carbofuran ring-C^{14} and 3-hydroxy carbofuran-ring-C^{14}, were individually incubated with Takamine Cellulase. The hydrolytic products after acetylation gas chromatographed as shown in Figure 6. Glycoside I is converted to glycoside H and free 3-hydroxycarbofuran. Cellulase treatment also resulted in the liberation of the 3-keto-7-phenol and the 3,7-diol. The glycosides of these phenols chromatographed between 3-hydroxycarbofuran and its glycoside.

DISCUSSION

Carbofuran is readily taken up through the roots of the alfalfa and bean plants. For the metabolism study, alfalfa plants were removed from field plots and replanted into pots during the late fall and early winter. The amount of top growth obtained from these plants in the greenhouse during the winter was adequate for these studies. The C^{14} residues were determined by combustion and C^{14} liquid scintillation counting procedures. Four hundred grams of alfalfa hay containing carbofuran-carbonyl-C^{14} residues (76 ppm) and 600 g of hay containing carbofuran-ring-C^{14} residues (306 ppm) were made available from these studies for residue metabolism studies in the dairy cow and rat.

Diethyl ether was used in the extraction studies to remove crude fat prior to the extraction of the glycosides, free phenols, and carbamates with methanol. Ring labeled radioactive materials not extracted with methanol are believed to be insoluble glycosides.

Silica gel columns were used to separate the metabolites extracted by diethyl ether and methanol. Except for carbofuran and the 3,7-diol, the silica gel column separated the free phenols and carbamates. All the glycosides chromatographed as sharp symmetrical peaks except for G. On rechromatography this glycoside partially chromatographed as H. The glycoside appeared to be bound to cellular materials.

For characterization purposes, glycosides G, H, and I were acid hydrolyzed and their aglycones identified and quantitated by chromatography and liquid scintillation counting. Identical aglycones in varying percentages were found. The studies suggested that 3-hydroxycarbofuran, the 7-phenol, the 3-keto-7-phenol, and the 3,7-diol were either conjugated in the plant with different monosaccharides or where conjugated with mono-, di-, and trisaccharides of similar composition.

The glycosides of the major aglycones, 3-hydroxycarbofuran, were examined by gel filtration and gas chromatography to determine whether the glycosidic residues were all monoglycosides. Glycoside H, the first glycoside to chromatograph on silica gel as a sharp peak, gas chromatographed when acetylated as a single component. Glycosides I and J did not gas chromatograph. On Bio-Gel P-2, glycoside I eluted first, followed by glycoside H, while glycoside J eluted in the same position as H. The results indicated that the glycosidic residues of these glycosides were monosaccharide (H and J) and disaccharide (I).

To further substantiate these facts, the carbofuran-carbonyl-C^{14} metabolites from alfalfa were incubated with an *Aspergillus niger* cellulase preparation from Miles Laboratories (Takamine Cellulase 4000). The preparation is active against a number of β-D-glucopyranosides (Li and King, 1963) and gentibiose. Glycoside I (carbonyl-C^{14} label) was hydrolyzed by the cellulase preparation to 3-hydroxycarbofuran and possibly to glycoside H. Hydrolytic studies with ring-C^{14} labeled glycoside(s) I from the bean plant showed that this glycoside(s) is converted to glycoside(s) H by enzymatic hydrolysis. The nature of the sugars involved and their linkages are unknown. The literature indicates that D-glucose is the major sugar involved in the glycosylation of phenols and the linkages usually are β. Di-glycosides are formed by further glycosylation at the 4 or 6 position of the first glucose residue.

The gel filtration study indicated that glycoside J was most likely a monoglycoside even though it did not gas chromograph after acetylation. Phenols conjugated with glucuronic acid have been reported. Glycosides G and H from bean plants individually administered carbonyl-C^{14} labeled carbofuran and 3-hydroxycarbofuran were examined by DEAE-cellulose chromatography (Knaak *et al.*, 1965). Direct feeding of bean plants with 3-hydroxy carbofuran resulted in the formation of an anionic glycoside (40%), while only 2% of the glycosides of 3-hydroxy carbofuran were anionic when carbofuran was fed. The glycosides from alfalfa were not examined by ion exchange chromatography.

Figure 7 gives the proposed pathway for the metabolism of carbofuran in alfalfa using glucose as the simplest conjugating sugar.

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