Persistent Glycosides of Metabolites of Methylcarbamate Insecticide Chemicals Formed by Hydroxylation in Bean Plants

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Studies with eight substituted phenyl methylcarbamate and two substituted pyrazolyl dimethylcarbamate insecticide chemicals indicate that the water-soluble metabolites formed from them, following injection into bean plants, result in part from hydroxylation of the carbamate on the *N*-methyl group, on the ring, or on a ring substituent, followed by conjugation of the hydroxylated carbamates, mainly as glycosides. These glycosides are quite persistent and, in many cases, yield anticholinesterase agents on hydrolysis by β-glucosidase. The aglycones derived from carbaryl, which include the *N*-hydroxymethyl, 4-hydroxy, 5-hydroxy, and 5,6-dihydro-5,6-dihydroxy derivatives, are the same carbamate intermediates involved in carbaryl metabolism by mammals and insects. *N*-Hydroxymethyl formation also occurs with Banol, Baygon, and UC 10854. Hydroxylation of the tertiary carbon of the isopropyl group yields hydroxypropyl UC 10854 from UC 10854, and 2-hydroxyphenyl methylcarbamate on *O*-depropylation of Baygon. Horseradish peroxidase degrades Matacil and Zectran while tyrosinase systems do not metabolize the four carbamates studied.

A recent publication showed that aryl methylcarbamate insecticide chemicals are metabolized into organo-soluble products, water-soluble products, and insoluble residues following injection into the stem of growing bean plants (Abdel-Wahab *et al.*, 1966). The water-soluble metabolites varied with the compound, were persistent in the plant, and after three to six days represented the following percentages of the 25-μg. injected dose:

Compound	Chemical Name	Water-Soluble Metabolites, %
Banol	2-Chloro-4,5-xylyl methylcarbamate	45–66
Baygon	2-Isopropoxyphenyl methylcarbamate	59–68
Carbaryl	1-Naphthyl methylcarbamate	35–39
Dimetilan	1-Dimethylcarbamoyl-5-methyl-3-pyrazolyl dimethylcarbamate	16–25
HRS-1422	3,5-Diisopropylphenyl methylcarbamate	12–23
Isolan	1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate	20–26
Matacil	4-Dimethylamino-3-cresyl methylcarbamate	7–8
Mesurol	4-Methylthio-3,5-xylyl methylcarbamate	8–9
UC 10854	3-Isopropylphenyl methylcarbamate	56–64
Zectran	4-Dimethylamino-3,5-xylyl methylcarbamate	4–5

The studies with dimetilan and Isolan were made at a different time of year than the studies with the methylcarbamates; therefore, the studies for the two types of carbamates are not necessarily directly comparable (Kuhr, 1966). Mesurol is oxidized in beans to 4-methylsulfinyl- and 4-methylsulfonyl-3,5-xylyl methylcarbamates, while Zectran and Matacil are oxidized selectively, at the dimethylamino grouping, to yield certain or all of the corresponding 4-methylformamido, 4-methylamino, 4-formamido, and 4-amino analogs (Abdel-Wahab *et al.*, 1966).

In related studies with cotton plants, the major carbaryl metabolite following absorption of carbaryl through the roots contains the COC(O)NC moiety intact and, although not identified, is probably produced by hydroxylation of the naphthalene ring (Mostafa *et al.*, 1966). The systemic carbamate, 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime, is converted in cotton to sulfoxide and sulfone analogs and the oxime resulting from hydrolysis of the sulfoxide analog (Metcalf *et al.*, 1966a).

This report concerns additional studies with beans made to determine the nature of the water-soluble metabolites of carbamate insecticides, particularly those derived from Banol, Baygon, and carbaryl. Included in these studies were experiments made with horseradish peroxidase and mushroom tyrosinase to determine whether or not these enzyme systems would, under in vitro conditions, degrade these methylcarbamates, in part, according to the chemical scheme found for them in the bean plant.

MATERIALS, APPARATUS, AND TEST CONDITIONS

The pure radioactive and nonradioactive compounds and their sources are given in Table I or in a corresponding table in a previous publication (Abdel-Wahab *et al.*, 1966). Each of the radioactive compounds was of a radiochemical purity of 99% or better, and each had an adjusted specific activity of 1.0 mc. per mmole after dilution with the respective pure, unlabeled compound. The enzymes and coenzymes and their sources are as follows: β-glucosidase (B grade), horseradish peroxidase (A grade), nicotinamide adenine dinucleotide (NAD) and its reduced form ($NADH_2$), nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form ($NADPH_2$) from Calbiochem, Los Angeles, Calif., glucose oxidase (pure), mushroom tyrosinase (lyophilized), and β-glucuronidase (salt free) from Nutritional Biochemical

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Table I. Name, Chemical Name, and Source of Compounds

Compound	Chemical Name	Source
Banol *N*-methyl-C^{14}	2-Chloro-4,5-xylyl methylcarbamate	Upjohn Co., Kalamazoo, Mich.
Baygon isoprop-1,3-C^{14}-oxy	2-Isopropoxyphenyl methylcarbamate	World Health Organization, Geneva, Switzerland
Baygon *N*-methyl-C^{14}		Krishna and Casida, 1966
Carbaryl *N*-methyl-C^{14}	1-Naphthyl methylcarbamate	Krishna and Casida, 1966
Carbaryl naphthyl-1-C^{14}		Leeling and Casida, 1966
O-Depropyl Baygon	2-Hydroxyphenyl methylcarbamate	Balba, 1967
Dihydrodihydroxy carbaryl carbonyl-C^{14}	5,6-Dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate	Oonnithan and Casida, 1966
Dimetilan carbonyl-C^{14}	1-Dimethylcarbamoyl-5-methyl-3-pyrazolyl dimethylcarbamate	Geigy Research Laboratories, Yonkers, N.Y.
4-Hydroxy Baygon	2-Isopropoxy-4-hydroxyphenyl methylcarbamate	Balba, 1967
4-Hydroxy carbaryl	4-Hydroxy-1-naphthyl methylcarbamate	Knaak *et al.*, 1965
5-Hydroxy carbaryl	5-Hydroxy-1-naphthyl methylcarbamate	Balba, 1967
4-Hydroxy Zectran	4-Hydroxy-3,5-xylyl methylcarbamate	Balba, 1967
N-Hydroxymethyl Banol	2-Chloro-4,5-xylyl *N*-hydroxymethylcarbamate	Balba, 1967
N-Hydroxymethyl Baygon	2-Isopropoxyphenyl *N*-hydroxymethylcarbamate	Balba, 1967
N-Hydroxymethyl carbaryl	1-Naphthyl *N*-hydroxymethylcarbamate	Balba, 1967
N-Hydroxymethyl Matacil	4-Dimethylamino-3-cresyl *N*-hydroxymethylcarbamate	Balba, 1967
N-Hydroxymethyl UC 10854	3-Isopropylphenyl *N*-hydroxymethylcarbamate	Balba, 1967
N-Hydroxymethyl Zectran	4-Dimethylamino-3,5-xylyl *N*-hydroxymethylcarbamate	Balba, 1967
Hydroxypropyl UC 10854	3-[2-(2-hydroxypropyl)]phenyl methylcarbamate	Balba, 1967
Isolan carbonyl-C^{14}	1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate	Geigy Research Laboratories
Methyl-C^{14}-amine		Nuclear Chicago Corp., Des Plaines, Ill.
1-Naphthol-1-C^{14}		Nuclear Chicago Corp.

Corp., Cleveland, Ohio, glusulase (100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml.) from Endo Laboratories, Inc., Garden City, N.Y.

The materials, apparatus, and test conditions used for growing the bean plants, thin-layer chromatography, detection of unlabeled compounds on the chromatoplates, radioautography, radioactivity counting, etc. were the same as the applicable ones described by Abdel-Wahab *et al.* (1966), with certain exceptions and changes as noted elsewhere in this report. The solvent systems and types of silica gel used in thin-layer chromatography (TLC) were as follows:

Type of Compound	Silica Gel	Solvents
Methyl- and dimethylcarbamates	G	Chloroform-acetonitrile (4:1)
Methylcarbamates	G	Ether-hexane (4:1)
Dimethylcarbamates	G	Ethyl acetate-ethanol (98:2)
Conjugates of methylcarbamate metabolites derived from:		
Banol	H	Ethyl acetate-isopropanol-water (64:24:12)
Baygon	H	Upper phase from *n*-butanol-water-acetic acid (50:48:2)
Carbaryl	H	Ethyl acetate-2-propanone-water-formic acid (5:3:1:1)

The silica gel was obtained from Kensington Scientific Corp., Berkeley, Calif., and a 0.5-mm. thickness was generally used. In making the radioautographs, the film was exposed for three to seven days at 5° C. or −10° C., the low temperature being used only in the case of experiments involving anticholinesterase assays on the resolved products. For cochromatography, approximately 20 μg. of each known, unlabeled compound, suspected to be identical in structure with a metabolite, was added to a solution containing a mixture of labeled metabolites before the solution was spotted on the plate. The following types of samples were used in the radioactivity counting: 0.2-ml. aliquots of aqueous fractions; 0.5- to 2.0-ml. aliquots of organic extracts from which the solvent was removed by evaporation; $C^{14}O_2$ trapped in the combustion of the unextractable fractions from the plant (Abdel-Wahab *et al.*, 1966; Krishna and Casida, 1966); scrapings of the radioactive gel regions of the TLC plates.

All of the tabulated results are based on radioactivity counting. They are the average of three experiments except in the following cases: only two experiments were made for the purpose of determining the amount of hydrolysis, achieved by enzymes and acid, of the water-soluble materials obtained from plants treated with the variously labeled carbaryl preparations (Table III), and for the detection of anticholinesterase agents (Table VI). The term μg. equivalent is the microgram level of metabolite expressed in terms of the parent compound based on equivalent C^{14} content.

METHODS

Treatment of Plants and Extraction of Metabolites. As previously described, 25-μg. portions (approximately 50,000 c.p.m.) of each of three radioactive carbaryl preparations containing C^{14} in the carbonyl, *N*-methyl, or naphthyl-1 position, as well as an approximately equivalent amount (50,000 c.p.m.) of C^{14}-labeled 1-naphthol, methylamine, and sodium carbonate, were individually injected into the stems of 11-day-old, live bean plants (Abdel-Wahab *et al.*, 1966). After the injected, growing plants had been held in the greenhouse for 0, 1, 3, and 6

days, respectively, each set of three identically treated plants was cut, stored, and extracted with acetone and chloroform, using the described procedure. This divided the components into three fractions: the acetone-chloroform or organic phase, the acetone-water or aqueous phase, and the insoluble or unextractable residue. The total radioactivity of each fraction was determined by the previously published procedure (Abdel-Wahab *et al.*, 1966). The per cent of radioactivity found in each fraction was calculated, and that not accounted for was entered as loss. The results are given in Table II.

In subsequent experiments made to obtain a larger amount of water-soluble products, 100 μg. (approximately 200,000 c.p.m.) of each of the following radioactive compounds in 40 μl. of acetone-water mixture (1:1) were individually injected into 11-day-old bean plants.

Banol carbonyl-C^{14}	Dimetilan carbonyl-C^{14}
Banol *N*-methyl-C^{14}	HRS-1422 carbonyl-C^{14}
Baygon carbonyl-C^{14}	Isolan carbonyl-C^{14}
Baygon isoprop-1,3-C^{14}-oxy	Matacil carbonyl-C^{14}
Baygon *N*-methyl-C^{14}	Mesurol carbonyl-C^{14}
Carbaryl carbonyl-C^{14}	UC 10854 carbonyl-C^{14}
Carbaryl *N*-methyl-C^{14}	Zectran carbonyl-C^{14}
Carbaryl naphthyl-1-C^{14}	

The injected plants were held for 6 days in the greenhouse, and individually extracted, analyzed, and counted as previously described, except that 15 ml. (instead of 10 ml.) of water was added to the organic extract to separate the water-soluble fraction (Abdel-Wahab *et al.*, 1966). The 100-μg. dosage was used in all the studies except those presented in Tables II and III.

Table II. Fractions Recovered from Growing Bean Plants Injected with C^{14}-Labeled Carbaryl or with Its C^{14}-Labeled Hydrolysis Products, and Sampled at Intervals after Treatment in October 1965

Fraction	Per Cent of Injected Radioactivity Found for Indicated Time, in Days, after Treatment			
	0	1	3	6
Carbaryl naphthyl-1-C^{14}				
Organic phase	77.1	58.0	27.8	1.0
Aqueous phase	1.6	10.5	22.6	36.9
Insoluble residue	0.6	8.8	20.4	29.5
Loss	20.7	22.7	29.2	32.6
Carbaryl carbonyl-C^{14}				
Organic phase	79.3	53.9	22.6	2.8
Aqueous phase	0.5	10.4	23.1	30.4
Insoluble residue	0.8	7.8	12.4	18.0
Loss	19.4	27.9	41.9	48.8
Carbaryl *N*-methyl-C^{14}				
Organic phase	74.7	57.5	28.4	4.4
Aqueous phase	0.0	10.4	23.7	37.0
Insoluble residue	0.5	7.3	15.5	22.2
Loss	24.8	24.8	32.4	36.4
1-Naphthol-1-C^{14}				
Organic phase	1.8	0.4	0.2	0.3
Aqueous phase	1.0	5.5	6.1	5.1
Insoluble residue	53.9	71.9	72.6	67.0
Loss	43.3	22.2	21.1	27.6
Sodium carbonate-C^{14}				
Organic phase	0.0	0.7	1.2	0.5
Aqueous phase	3.4	5.5	1.3	1.2
Insoluble residue	11.3	60.8	51.4	57.3
Loss	85.3	33.0	46.1	41.0
Methyl-C^{14}-amine				
Organic phase	0.1	0.1	0.8	1.0
Aqueous phase	53.7	38.3	22.0	11.9
Insoluble residue	23.2	32.7	36.6	48.8
Loss	23.0	28.9	40.6	38.3

Enzymatic Cleavage of Water-Soluble Conjugates. Four to 15 ml. of the aqueous phase, containing the water-soluble metabolites, were placed in a 25-ml. Erlenmeyer flask, and the water was evaporated under reduced pressure on a rotary evaporator at a water bath temperature of 45° C. The residue was treated with 4 ml. of citrate-phosphate buffer (pH 4.4, made by mixing 0.1*M* citric acid and 0.2*M* disodium phosphate solutions) and, in individual experiments, with one of the following: no addition, 3 mg. of β-glucosidase, 3 mg. of β-glucuronidase, 100 μl. of glusulase solution, hydrochloric acid to a final concentration of 9% (w./v.), or hydrochloric acid to a final pH of 1.0. The flasks were shaken at 38° C., in air, for 4 to 5 hours. The contents of the flask were poured into a 15-ml. centrifuge tube and extracted three times with 5-ml. portions of ether. After combining the ether extracts, the radioactivity of the ether and aqueous fractions was measured. The ether was evaporated to dryness with a stream of nitrogen, the residue was dissolved in 200 μl. of acetone and, in certain cases, suitable amounts of known unlabeled compounds (suspected metabolites) were added. The entire solution was spotted on a TLC plate and after development in ether-hexane mixture and radioautography, the radioactive content of each resolved metabolite was determined and, when possible, some of the products were tentatively identified by cochromatography.

Separation of Radiolabeled Water-Soluble Metabolites. Approximately 15 ml. of each water phase obtained from treated plants were evaporated to dryness under reduced pressure (as above), 400 μl. of a water-ethanol mixture (1:1) were added, and the entire soluble portion was spotted on a TLC plate as a series of small, separate spots as a band along the origin. After development in the solvent system appropriate for conjugates of the compound under study (see above) and radioautography, each radioactive region was scraped from the plate into a 15-ml. centrifuge tube, and the gel was extracted with two 10-ml. portions of methanol. The methanol extracts were combined, the radioactivity of an aliquot was counted, and the remaining portion was evaporated to dryness in a 25-ml.-Erlenmeyer flask. Buffer, or buffer containing β-glucosidase, was added, the mixture was incubated, and the

Table III. Enzymatic and Acidic Cleavage of Carbaryl-C^{14} Water-Soluble Metabolites Recovered from Growing Bean Plants Six Days after Injection with Variously Labeled Carbaryl-C^{14} Preparations

Cleavage Conditions	pH	Cleavage, %[a]		
		Naphthyl-1-C^{14}	Carbonyl-C^{14}	*N*-Methyl-C^{14}
Buffer only (control)	4.4	7	5	6
β-Glucosidase and buffer	4.4	55	50	47
Glusulase and buffer	4.4	61	61	47
Hydrochloric acid	1.0	33	25	32

[a] Percentage of total radiocarbon recovered in ether relative to that in ether plus water phases, after incubation under stated cleavage conditions.

ether-extractable products were subjected to TLC analysis, as above.

Anticholinesterase Assays of Organosoluble Metabolites. Water-soluble fractions from bean plants individually treated with each of the eight methylcarbamates carbonyl-C^{14} and two dimethylcarbamates carbonyl-C^{14} were incubated with buffer alone or with buffer plus β-glucosidase, using the conditions given above. After incubation, the ether-extractable products were separated on TLC plates coated with silica gel G of 0.25-mm. thickness. Development in the first direction was with chloroform-acetonitrile mixture, and in the second direction was with ether-hexane mixture for the methylcarbamates or with ethyl acetate–ethanol mixture for the dimethylcarbamates. Following radioautography, regions with cholinesterase-inhibition activity were detected as red spots on a yellow background directly on the plates, using the procedure of Oonnithan and Casida (1966). The plates were examined by intercomparisons in such a way as to locate definitely cholinesterase-inhibiting spots that were caused by naturally occurring materials present in the aqueous fraction of the plant extract and radioactive and nonradioactive materials released from the aqueous fraction on incubation with β-glucosidase. Finally, the radioactivity in each of the spots of interest was determined by scraping and scintillation counting.

Effect of Horseradish Peroxidase and Tyrosinase. Twenty-five micrograms of each of the 10 carbonyl-labeled methyl- and dimethylcarbamates and an approximately equivalent amount of sodium carbonate-C^{14} (50,000 c.p.m.) were individually incubated at 38° C. in air for one hour with 4 ml. of phosphate buffer (pH 7.4, 0.025*M*), 30 mg. of glucose, 100 μg. of glucose oxidase, and 100 μg. of horseradish peroxidase (HRP). Controls, involving omission of HRP only, were carried along in all studies and, in the case of Matacil and Zectran, the relative effectiveness of various HRP levels was determined.

In a buffered aqueous solution, 1 mg. of tyrosinase was incubated individually at 38° C. in air for one hour with 25 μg. of each of carbonyl-labeled samples of Banol, Baygon, carbaryl, and Matacil under conditions where tyrosinase has cresolase activity and under other conditions where it has catecholase activity (Dawson and Magee, 1955; Kuhr, 1966). The activity of the enzyme was ascertained to be high, using cresol and catechol as the substrates. In an additional study, the stability of each of the carbamates was determined when 25 μg. of them plus 4 mg. of NAD, $NADH_2$, NADP, or $NADPH_2$ were added to the cresolase-activity system.

In all cases, after the incubation step, the peroxidase or tyrosinase incubation mixtures were extracted with ether, and the radioactivity of the ether and water phases was counted. Finally, the radioactive products in the ether phase were separated by TLC and, where feasible, they were identified by cochromatography.

RESULTS

Fate of Radiocarbon in Variously Labeled Carbaryl-C^{14} Samples and Their Hydrolysis Products. The position of the radiocarbon in carbaryl-C^{14}, whether in the carbonyl, *N*-methyl, or 1-naphthyl position, has only a small effect on its distribution among the plant extract fractions at any time after injection of carbaryl samples into the bean plant (Table II). In every case, the organic phase contains only carbaryl and not any metabolites, based on two-dimensional TLC analysis with ether-hexane mixture; in view of this, the results obtained confirm the expectation that the labeling position would have little effect on the products in the organic phase. The naphthyl-labeled sample gives a larger amount of radioactivity in the insoluble portion after six days than the carbonyl- or *N*-methyl–labeled carbaryl samples, indicating that the ring fragment may, in part, be selectively incorporated into the insoluble residue following partial hydrolysis of the carbamate ester. Another indication that some hydrolysis of the carbamate ester group occurs in the plant is also afforded by the results with carbaryl carbonyl-C^{14}, where the over-all loss of radiocarbon, possibly as $C^{14}O_2$, is higher than with the carbaryl samples labeled in other positions. 1-Naphthol-1-C^{14} is rapidly incorporated into the insoluble residue, leaving little radioactivity in either the organic or aqueous phases throughout the test period. Sodium carbonate-C^{14} and methyl-C^{14}-amine result in about the same loss after one to six days, and both deposit a substantial amount of the radioactivity in the insoluble residue. The fact that a small amount of the C^{14} from carbaryl carbonyl-C^{14} appears in the insoluble residue, compared with the large amount from sodium carbonate-C^{14}, may indicate that CO_2 released slowly by hydrolysis of the carbamate is less efficiently incorporated into the unextractable portion than a massive dose of CO_2 released by the carbonate; so, much more $C^{14}O_2$ is lost. The large radiocarbon losses with naphthol and sodium carbonate at day 0 are probably artifacts associated with the procedures of injection and extraction, but this area was not further investigated (Kuhr, 1966).

Cleavage of Water-Soluble Metabolites Derived from Carbaryl-C^{14}. As shown in Table III, about 50% of the water-soluble metabolites, present six days after injection of carbaryl-C^{14} into bean plants, are cleaved by β-glucosidase or glusulase, and the position of the radiocarbon does not greatly alter the result. This similarity in the extent of enzymatic cleavage for each of the variously labeled samples suggests that the majority of the metabolites contain the carbamate moiety and that the water-soluble metabolites consist, in large part, of glycosides with a carbaryl metabolite as the aglycone portion. After incubation in the absence of enzyme, only 5 to 7% of the radiocarbon is recovered on extraction with ether, and variation of the labeling position has little or no effect; this indicates that the products are not readily cleaved nonenzymatically, at pH 4.4. However, at pH 1.0, the cleavage is increased to 25 to 33% indicating that certain of the water-soluble metabolites are acid labile. The loss of radioactivity during the above incubations and extractions averaged about 7%, even with carbaryl carbonyl-C^{14} metabolites. Under these incubation conditions, carbonate-C^{14} is almost completely lost, indicating that the products from the insecticide chemicals are carbamates rather than carbonate.

Nature and Chromatographic Characteristics of Water-Soluble Metabolites Derived from Various C^{14}-Labeled Banol, Baygon, and Carbaryl Preparations. Six days after injection of the radioactive preparations of Banol, Baygon,

and carbaryl into the stem of the bean plant, the amounts of water-soluble metabolites recovered, as a percentage of the amount of radiocarbon used, were as follows:

Radioactive Group	Banol	Baygon	Carbaryl
Carbonyl	12	36	16
N-Methyl	18	32	19
Naphthyl	...	...	18
Isopropoxy	...	10	...

With the exception of the isopropoxy-labeled form of Baygon, the amounts of water-soluble metabolites formed are approximately the same, regardless of the position of the radiocarbon. This suggests that the carbamate moiety [COC(O)NC] remains largely intact. As shown in Table IV, the water-soluble metabolites are resolvable by TLC into several regions, the number varying as follows: Banol, 2; Baygon, 5; carbaryl, 6. Unfortunately, there is only a sequential interrelationship between the TLC position of these regions because of the necessity of using different solvent systems to resolve the metabolites. With each compound, the carbonyl- and *N*-methyl–labeled preparations give roughly the same distribution of radioactivity in each TLC region; however, in the case of Baygon, the isopropoxy-labeled material gives a different radiocarbon distribution in each of the three major regions and, in the case of carbaryl, the naphthyl-labeled one shows this characteristic to some extent.

Cleavage by β-glucosidase of the water-soluble metabolites found in each of the TLC regions does not, on a percentage basis, reveal a distinct or discernible distribution pattern. However, in the case of carbaryl, three regions (A,D,E) show a greater cleavage when the radiocarbon is in the naphthyl group; in the case of Baygon, only one region (E) shows this same behavior with the isopropoxy-labeled group. With Baygon, distinctly low cleavage occurs with the isopropoxy-labeled sample in two regions (B,D). The cleavage of Banol metabolites is generally the same for both of the labeled groups used. Thus, a general pattern of per cent cleavage of the water-soluble metabolites does not emerge with the different labeled samples because the aromatic group or an *O*-alkyl substituent on this group may be removed on metabolism, as will be discussed later.

All three of the methylcarbamates used give a wide range in the amounts of aglycones recovered, after cleavage, from the water-soluble metabolites in various TLC regions (Table IV). Except in the case of two regions (E,F), the position of the radiocarbon in carbaryl does not importantly alter the aglycone recovery, either in the regions or in the total amount recovered. This is generally true for Banol, too (without a notable exception). However, in the case of Baygon, the recovery of aglycones with the isopropoxy-labeled material is erratic, being exceptionally low for the major TLC region (B) and relatively high in another region (C); in regard to the total recovered, the aglycones found with the isopropoxy-labeled preparation are approximately one-half of that for the carbonyl- and *N*-methyl-labeled materials. Thus, the isopropoxy group from about one-half of the Baygon is lost or forms non-cleavable water-soluble metabolites (which helps to explain the erratic results).

These findings fortify the suggestion that, during metabolism of these methylcarbamates in the plant, the carbamate moiety remains, in part, intact and they indicate that the water-soluble metabolites are mixtures of conjugated compounds and not single compounds. The carbamate moiety [COC(O)NC] is intact in the majority

Table IV. Chromatographic and Enzyme-Cleavage Characteristics of Water-Soluble Metabolites Recovered from Growing Bean Plants Six Days after Injection with Variously C^{14}-Labeled Samples of Carbaryl, Baygon, and Banol

Regions for Water-Soluble Metabolites Partially Resolved by TLC		Radiocarbon in Each Region from Different Labeled Samples					
		Per cent recovered in relation to total water-soluble metabolites			Per cent of products cleaved by β-glucosidase		
Designation	R_f Value						
Carbaryl-C^{14} metabolites		Naphthyl-1-C^{14}	Carbonyl-C^{14}	*N*-Methyl-C^{14}	Naphthyl-1-C^{14}	Carbonyl-C^{14}	*N*-Methyl-C^{14}
A	0.20	10	10	14	25	12	12
B	0.37 + 0.41	30	40	34	35	34	36
C	0.55	34	33	34	83	81	76
D	0.66	9	11	14	48	30	34
E	0.78	13	5	3	57	35	32
F	0.91	4	1	1	68	82	69
Baygon-C^{14} metabolites		Isoprop-C^{14}-oxy	Carbonyl-C^{14}	*N*-Methyl-C^{14}	Isoprop-C^{14}-oxy	Carbonyl-C^{14}	*N*-Methyl-C^{14}
A	0.29	2	3	3	27	54	28
B	0.62	34	83	85	27	58	63
C	0.73	32	7	5	46	60	47
D	0.77	31	5	5	8	25	23
E	0.78	1	2	2	87	68	58
Banol-C^{14} metabolites			Carbonyl-C^{14}	*N*-Methyl-C^{14}		Carbonyl-C^{14}	*N*-Methyl-C^{14}
A	0.17		28	30		16	15
B	0.70		72	70		56	46

Note: There is only a sequential interrelationship between the R_f values and metabolite designation of metabolites obtained from carbaryl, Baygon, or Banol because different solvent systems were utilized.

of the carbaryl water-soluble metabolites. Further, there is evidence that the fundamental skeleton of Baygon [2-C(C)CH—O—aryl—OC(O)NC] is present in a fraction of the plant-produced metabolites, but that more than half of them have been produced by cleavage of the isopropoxy grouping in one step or another of their formation. Since a preparation with a radiocarbon in the ring part of the molecule was not used, the evidence for Banol is limited, but it is clear that the C(O)NC moiety remains intact in the plant. This strongly suggests that the water-soluble metabolites of Banol contain moieties that are carbamate esters.

Aglycones Released by β-Glucosidase on Incubation of Water-Soluble Metabolites Derived from Several C^{14}-Labeled Methylcarbamates. Cleavage of materials by β-glucosidase in the water-soluble fractions (obtained from plants injected with carbonyl-C^{14} preparations of Banol, Baygon, and carbaryl) yielded ether-extractable products to the following extent: Banol, 80%; Baygon, 76%; carbaryl, 46%. Resolution and tentative identification of the ether-extractable products by cochromatography yield a variety of aglycones (Table V). A small amount (0.1 to 1.0%) of the parent compound and a varying amount of the *N*-hydroxymethyl analog are recovered in each case. In the case of carbaryl, the 4-hydroxy and 5-hydroxy analogs are present in important amounts. The carbaryl metabolite characterized as dihydrodihydroxy carbaryl is tentatively so designated because it cochromatographs with a compound known to have this structure as formed by the rat liver microsome-$NADPH_2$ enzyme system (Leeling and Casida, 1966). 2-Hydroxyphenyl methylcarbamate (*O*-depropyl Baygon) is the major aglycone formed from Baygon. A labeled material which apparently cochromatographs with 4-hydroxy Baygon is obtained; however, this product may not be 4-hydroxy Baygon, because authentic samples of compounds with other sites of ring hydroxylation (2-isopropoxy-3-hydroxyphenyl methylcarbamate; 2-isopropoxy-5-hydroxyphenyl methylcarbamate; 2-isopropoxy-6-hydroxyphenyl methylcarbamate) are not available for use as standards for cochromatography.

The major aglycones formed from Baygon and carbaryl are tentatively identified, but a number of minor products remain to be characterized. The major products formed from Banol are not, as yet, identified. The total losses suffered in chromatography of the ether extracts and radioactivity measurements are 23% for Banol and carbaryl and 37% for Baygon.

In a more limited study of UC 10854 metabolites, *N*-hydroxymethyl UC 10854 and hydroxypropyl UC 10854

Table V. Characteristics and Distribution of C^{14}-Labeled Aglycones Released by β-Glucosidase from Water-Soluble Metabolites Recovered from Growing Bean Plants Six Days after Injection with Carbonyl-C^{14}-Labeled Samples of Carbaryl, Baygon, and Banol

Source of Metabolites	Aglycone Formed			Frequency of Occurrence of Aglycone in Chromatographic Region of Conjugated Metabolite[a]					
	Designation	R_f Value	Amount[b]	A	B	C	D	E	F
Carbaryl carbonyl-C^{14}	Unknown	0.00	4.9	7	9	9	8	3	0
	Dihydrodihydroxy carbaryl	0.18	15.6	4	9	1	0	9	0
	Unknown	0.38	1.5						
	N-Hydroxymethyl carbaryl	0.52	18.1	3	4	5	1	1	0
	4-Hydroxy carbaryl	0.55	33.0	8	8	9	8	5	7
	5-Hydroxy carbaryl	0.60	25.4	9	8	9	9	2	0
	Unknown	0.71	0.5	4	5	0	0	0	1
	Carbaryl	0.76	1.0	4	5	0	0	1	3
	1-Naphthol	0.95	[c]	0	0	3[c]	0	3[c]	2[c]
Baygon carbonyl-C^{14}	Unknown	0.00	0.7	0	6	8	9	0	
	Unknown	0.29	0.2	0	4	0	0	0	
	Unknown	0.35	1.6	0	6	3	0	3	
	Unknown	0.40	0.3						
	Unknown	0.47	0.5	0	5	0	0	0	
	O-Depropyl Baygon	0.52	91.3	7	9	6	6	6	
	4-Hydroxy Baygon	0.56	0.6	0	0	6	2	0	
	N-Hydroxymethyl Baygon	0.59	4.4	0	1	9	9	5	
	Unknown	0.63	0.3	0	0	1	2	3	
	Baygon	0.85	0.1	0	0	0	0	4	
	Unknown	0.92	<0.1	0	0	0	0	3	
Banol carbonyl-C^{14}	Unknown	0.00	1.0	6	6				
	Unknown	0.08	0.8	0	2				
	Unknown	0.13	2.5	0	2				
	Unknown	0.25	0.7						
	Unknown	0.31	10.5	6	5				
	Unknown	0.40	83.2	6	5				
	Unknown	0.47	0.2						
	N-Hydroxymethyl Banol	0.55	1.0	6	3				
	Banol	0.69	0.1						

[a] See Table IV for R_f value of regions; for carbaryl and Baygon, a total of nine experiments equally distributed between carbonyl-, *N*-methyl-, or isopropoxy-labeled materials; for Banol, a total of six, distributed between carbonyl- and *N*-methyl–labeled materials.
[b] Per cent of sum of radioactivity in all spots.
[c] Contained radioactivity only in the studies with carbaryl naphthyl-1-C^{14}.

were identified by cochromatography among the products formed in bean plants. One day after treatment, the organosoluble fraction contained two unconjugated unknowns (III-A and III-B, Table V, Abdel-Wahab *et al.*, 1966); the former material was hydroxypropyl UC 10854 while the latter material appeared to be *N*-hydroxymethyl UC 10854. When the water-soluble fraction derived from plants six days after treatment was cleaved by β-glucosidase, the same two compounds were liberated but, in this case, *N*-hydroxymethyl UC 10854 was present in much larger amounts than hydroxypropyl UC 10854. [*N*-Hydroxymethyl UC 10854 is aglycone 5 and hydroxypropyl UC 10854 chromatographs between aglycones 3 and 4 (see Table XIII, Kuhr, 1966).] The other aglycones derived from UC 10854 were not identified (Kuhr, 1966).

When the substances recovered from each of the partially resolved water-soluble TLC metabolite regions are incubated with β-glucosidase, the cleavage generally yields aglycones of the same chemical nature, regardless of the R_f value of the regions yielding the metabolite (Table V). Thus, surprisingly, each of the major resolved water-soluble metabolite regions gives a mixture of aglycones which appear to be, for the most part, made up of the same compounds. Therefore, the resolved water-soluble metabolites must be mixtures of conjugates, of various types and combinations, of the same aglycones.

Table V shows that following cleavage of the water-soluble metabolites from each of the TLC regions by β-glucosidase, the location of the radiocarbon in carbaryl has little or no effect on the frequency of detection of the various identical aglycones; however, as expected, 1-naphthol is detected only when using carbaryl with the radiocarbon in the naphthyl group. Each of the identified aglycones, other than 1-naphthol, has the COC(O)NC moiety intact. In each of the six resolved regions, there are water-soluble metabolites which are conjugates of 4-hydroxy carbaryl; conjugates of 5-hydroxy carbaryl and *N*-hydroxymethyl carbaryl are present in five of the six resolved regions. On the other hand, conjugates of 1-naphthol appear only in three regions (C,E,F), and those of dihydrodihydroxy carbaryl occur in four regions, being most frequent in region A and, particularly, in regions B and E. The identity of the ether-extractable products which occur at R_f 0.00, 0.71, and 0.76 is not known; however, the one at R_f 0.76 may be carbaryl, but this has not been established by cochromatography. On the basis of attempted cochromatography, neither of the products with the higher R_f values is 1-naphthyl carbamate.

Baygon also yields a variety of aglycones on cleavage, with β-glucosidase, of the resolved water-soluble metabolites in the five TLC regions (Table V). The same aglycones are always detected, with about the same frequency, when using the carbonyl-C^{14} and *N*-methyl-C^{14} samples of Baygon; so, all of the aglycones are probably carbamates. The product at R_f 0.92 is not 2-isopropoxyphenol because it is detected with the carbonyl-C^{14} and *N*-methyl-C^{14} but not with the isoprop-C^{14}-oxy compounds. The isopropoxy-labeled sample gives only four detectable aglycones occurring at R_f values of 0.00, 0.52, 0.56, and 0.59; the other aglycones are not detectable, either because they are present in very minor amounts or because they lack the isopropyl group or radioactive fragments thereof. Baygon yields an unidentified radioactive product which, although it chromatographs in the approximate R_f region of *O*-depropyl Baygon, is known not to be this compound because it is formed also from isopropoxy-labeled Baygon. However, this unknown product is present in minor amounts and it seems possible that conjugates of *O*-depropyl Baygon appear in each resolved region of water-soluble metabolites, and in region A, this appears to be the only conjugated carbamate. Conjugates of *N*-hydroxymethyl Baygon are present in several metabolite regions, and conjugates of 4-hydroxy Baygon (or a closely related compound) are present only in two regions and then only in minor amounts. Only TLC region E yields aglycones with R_f values of 0.85 and 0.92, but these are not necessarily conjugates; possibly they are compounds not released by β-glucosidase. Baygon has an R_f value of 0.90 in the solvent system used to separate the conjugates (water-soluble metabolites). Therefore, traces of Baygon and related unconjugated material, remaining in the aqueous phase after extraction and removal of the organic phase, could carry through the separation procedure and show an apparent conjugate, even though none really existed.

The *N*-methyl- and carbonyl-labeled Banol samples each give the same aglycones on cleavage of the resolved water-soluble metabolites with β-glucosidase; as seen in Table V, four of them originate from both water-soluble metabolite TLC regions. *N*-Hydroxymethyl Banol is the only aglycone tentatively identified; it appears only in small amounts but in both water-soluble metabolite TLC regions. The major metabolite, at R_f value of 0.40, which appears in both regions, is of unknown structure. However, based on analogy with an unpublished study made in this laboratory with a related compound, this major Banol metabolite may well be 2-chloro-4-hydroxymethyl-5-methylphenyl methylcarbamate.

Abundance and Anticholinesterase Activity of Aglycones Derived from Ten Methyl- and Dimethylcarbamates. The water-soluble fractions derived from plants treated with various carbonyl-C^{14}-labeled methyl- and dimethylcarbamates yield mixtures of radioactive aglycones when cleaved by incubation with β-glucosidase. The extent of cleavage, as a percentage of the total radiocarbon originally injected into the plant and of the total radiocarbon in the aqueous fraction, varies greatly with the different compounds (Table VI). Conjugates cleaved by β-glucosidase in highest amount are derived from Banol, Baygon, carbaryl, Isolan, and UC 10854, and those derived from the first four of these compounds are the most readily cleaved. The least tendency to be cleaved is found with conjugates from HRS-1422. The radioactive products released on incubation with buffer solution alone, in the absence of β-glucosidase, amounted to less than 3% with all compounds tested except HRS-1422, Matacil, and Zectran which release 8, 8, and 6%, respectively; these cleaved products remain at the origin during chromatography. However, incubation of the water-soluble fractions with β-glucosidase releases many radioactive substances, most of which move free of the origin during thin-layer chromatography. When the aqueous fractions from untreated plants and carbamate-treated plants

Table VI. Abundance and Anticholinesterase Activity of Radioactive Aglycones Released by β-Glucosidase from Water-Soluble Metabolites Recovered from Growing Bean Plants Six Days after Injection with 10 Carbonyl–C^{14}-Labeled Carbamate Insecticide Chemicals

Compound	C^{14}-Labeled Aglycones % Released in Relation to		Number of Radioactive and AntiChE Aglycones Recovered at Indicated μg. Levels, Expressed in Terms of Parent Compound Based on Equivalent C^{14} Content							
			<0.1		0.1–1.0		1.1–10		11–28	
	Radio-activity injected into plants	Total radio-activity in aqueous fraction	Radio-active	Anti-ChE	Radio-active	Anti-ChE	Radio-active	Anti-ChE	Radio-active	Anti-ChE
Banol	31	87	1	0	4	3	2	2	1	1
Baygon	33	69	3	1	2	0	1	0	1	1
Carbaryl	16	57	2	0	2	0	4	4		
Dimetilan	7.6	26	2	0	3	0	2	0		
HRS-1422	1.4	11	1	1	3	2				
Isolan	16	67	5	2					1	1
Matacil	1.9	25			5	0				
Mesurol	1.5	28	1	0	3	0				
UC 10854	14	27			4	2	3	3		
Zectran	1.2	27	1	0	4	2				

are extracted with ether in the absence of β-glucosidase in the incubation mixture, one or two cholinesterase-inhibiting TLC spots are obtained at identical R_f values in both cases. These aforementioned spots, plus one to three additional spots, occur for untreated plants when β-glucosidase is included in the incubation mixture (the spots probably result from natural phenolic compounds). Fortunately, none of these inhibitory spots cochromatograph with the radioactive regions, when present. Each additional inhibitory spot from carbonyl-C^{14} carbamate-treated plants, as released on incubation with β-glucosidase, cochromatographs with a radioactive spot; thus, the β-glucosidase incubation releases a series of aglycones, all of which are carbamates and many of which also are cholinesterase inhibitors.

The major carbamate aglycones from each of the 10 methyl- and dimethylcarbamates tested show anticholinesterase activity on the TLC plates, with the exception of those derived from Matacil and Mesurol which are present on the TLC plates only at low levels (Table VI). R_f values and quantitative data on each individual aglycone derived from the 10 carbamates are tabulated in thesis form (Kuhr, 1966). More than half of the radiolabeled aglycones from Banol, HRS-1422, and UC 10854 are anticholinesterase agents. Radioactive materials remaining at the origin of the developed TLC plates appear with each of the compounds; however, not any of these materials are cholinesterase inhibitors, and, possibly, these products are unhydrolyzed conjugates. Several of the aglycones, which move free of the origin, are detectable as anticholinesterase agents at levels of less than 0.1 μg., expressed in terms of the parent compound based on equivalent C^{14} content. It is not known whether or not other aglycones, detected as anticholinesterase agents at higher carbamate-equivalent levels, are of similar potency, because they were not compared at the lower equivalent levels. The number of aglycones shown in Table VI is probably a minimum number because only those aglycones that are completely resolvable by thin-layer chromatography are tabulated.

Action of Horseradish Peroxidase and Tyrosinase on Carbamates. Of the 10 carbonyl–C^{14}-labeled carbamates tested, only Matacil and Zectran were attacked during incubation with horseradish peroxidase (HRP). Ten micrograms of HRP, acting for one hour, result in 60% conversion of Zectran and 98% conversion of Matacil to products not recovered on extraction with ether. Even 0.5 μg. of HRP gives extensive Matacil degradation (Kuhr, 1966). Several of the oxidation products resulting from HRP action (not present in controls where only the HRP is omitted) appear, on the basis of cochromatography of the ether extracts, to be the following: from Zectran, the 4-methylamino and 4-amino derivatives which are always detected, and the 4-formamido and 4-methylformamido derivatives which are occasionally noted; from Matacil, the 4-methylamino derivative, present in all tests, and the 4-amino derivative, which is occasionally detected. No 4-hydroxy Zectran, *N*-hydroxymethyl Zectran, or *N*-hydroxymethyl Matacil is produced by HRP from Zectran and Matacil, respectively.

Although the water-soluble products formed from Zectran by the action of HRP have not been identified, the information available on them indicates that they are carbamates, that they are not *N*-hydroxymethyl, 4-hydroxy, 4-methylamino, 4-amino, 4-methylformamido, or 4-formamido Zectran, and that they probably are not derivatives resulting from reaction of carbamate intermediates with the glucose present in the reaction mixtures (Kuhr, 1966).

Banol, Baygon, carbaryl, and Matacil are not degraded, to any extent, in the tyrosinase systems, either under conditions for cresolase or catecholase activity, or on addition of pyridine nucleotide cofactors under the cresolase activity conditions.

DISCUSSION

The available evidence indicates that the water-soluble metabolites of substituted phenyl methylcarbamates and substituted pyrazolyl dimethylcarbamates in plants are, in

large part, conjugates of hydroxylated compounds with the carbamate ester group intact. The radiocarbon from carbaryl samples labeled in three different positions shows roughly the same distribution among the various metabolite fractions, whereas the distribution pattern of the corresponding labeled hydrolysis products (naphthol, carbonate, and methylamine) among the fractions is quite different (Table II). Approximately the same amount of enzymatic and acidic hydrolysis occurs with water-soluble metabolites derived from carbaryl preparations containing the radiocarbon, in separate preparations, in three different positions (Table III), and the same result occurs when the water-soluble metabolites are partially resolved by chromatography prior to cleavage by β-glucosidase (Table IV). Tentative identification of the aglycones shows that prior to conjugation carbaryl is hydroxylated at the *N*-methyl position (*N*-hydroxymethyl carbaryl) or on the ring (4-hydroxy carbaryl, 5-hydroxy carbaryl, and dihydrodihydroxy carbaryl) (Table V). Since anticholinesterase agents are released from the water-soluble metabolites on incubation with β-glucosidase, several carbamates must be present as conjugates (Table VI). Although more limited in scope, similar lines of evidence indicate that hydroxylation products of Baygon, Banol, and seven other methyl- and dimethylcarbamates are conjugated as glycosides which make up a high proportion of the water-soluble metabolite fraction (Tables IV–VI).

The rate-limiting reaction in bean plants must be hydroxylation of the carbamates rather than glycoside formation, because unconjugated hydroxylation products are present in small amounts, if at all, in the plant (Abdel-Wahab *et al.*, 1966). The organosoluble fraction from plants treated separately with Dimetilan and Isolan consists of a single labeled compound and this probably is the compound injected rather than an unconjugated metabolite (Kuhr, 1966). Evidence for glycosides is based mainly on cleavage of the water-soluble metabolites by β-glucosidase and extraction of the released aglycones into ether. In those cases where large amounts of hydroxylated carbamates are released, the liberated fragment is probably the aglycone portion of a glycoside. However, in other cases where aglycone-like products are detected in small amounts or with low frequency (following the action of β-glucosidase), the enzyme may have served only to release the aglycone from binding or combination with other plant water-soluble materials. Also, some of the aglycones may be metabolites not extracted from the original water phase by acetone-chloroform, as probably is the case with Baygon from conjugate region E (Table V). Finally, some of the released aglycones may be of low solubility in ether and not completely recovered in ether following cleavage by β-glucosidase.

Quantitative information on the aglycones indicates that conjugates of carbamate metabolites of Banol, Baygon, carbaryl, Isolan, and *UC* 10854 constitute 14 to 33% of the initial injected dose (Table VI). When calculated on the same basis as in Table VI but further defined on the basis of the individual aglycones (Table V), the amounts of aglycones-C^{14} present in bean plants 6 days after injection of 100 μg. of Banol carbonyl-C^{14}, Baygon carbonyl-C^{14}, and carbaryl carbonyl-C^{14} are as follows:

Metabolite		C^{14}-Labeled Aglycones, μg., Expressed in Terms of Parent Compound Based on Equivalent C^{14} Content
Banol		
N-Hydroxymethyl		0.3
Major unknowns (2)		29.2
Minor unknowns (5)		1.6
	Total	31.1
Baygon		
O-Depropyl		30.2
4-Hydroxy		0.2
N-Hydroxymethyl		1.5
Unknowns (6)		1.2
	Total	33.1
Carbaryl		
Dihydrodihydroxy		2.5
4-Hydroxy		5.2
5-Hydroxy		4.0
N-Hydroxymethyl		2.9
Unknowns (3)		1.1
	Total	15.7

Each of these metabolites probably contains the COC(O)NC skeleton intact; so, as expected, similar results are found with the carbonyl- and *N*-methyl–labeled samples. The isoprop-C^{14}-oxy sample of Baygon gives much different results than the carbonyl- and *N*-methyl–labeled samples; this is explained by the metabolism of 30% of the injected dose to *O*-depropyl Baygon, which would not be radiolabeled when derived from isopropyl-labeled Baygon. Acetone-C^{14} probably is the product from cleavage of the isoprop-C^{14}-oxy grouping because acetone has been demonstrated as the cleavage product in houseflies (Shrivastava, 1967). As expected, the water-soluble metabolites are lower in terms of percentage of radioactivity injected into the plant but higher in terms of micrograms, expressed as the parent compound based on equivalent C^{14} content, when using a 100-μg. dose per plant (Table VI) than with a 25-μg. dose (see Table V, Abdel-Wahab *et al.*, 1966).

The losses experienced in the process of evaporation, chromatography, and recovery of the separated water-soluble metabolites averaged 23% for all experiments with carbaryl (independent of the labeling position), 16% for those with Baygon (independent of the labeling position), 10% for Banol carbonyl-C^{14}, and 20% for Banol *N*-methyl-C^{14}. In the cleavage experiments, the losses suffered in the incubation and extraction steps were, on the average, 10% with the carbaryl materials with little variation with the position of the radiocarbon, 5% with Baygon carbonyl-C^{14} and Baygon *N*-methyl-C^{14}, 29% with Baygon isoprop-C^{14}-oxy, and 47% with the Banol materials, the largest loss being experienced with the material having the highest R_f value. In the case of Banol, the actual amount of cleavage was possibly as high as 90% because of the large losses suffered during the incubation in two of the three experiments. This large loss with Banol carbonyl-C^{14} was possibly related to the ease of nonenzymatic hydrolysis of Banol (Abdel-Wahab *et al.*, 1966) or it metabolites, the aglycones formed giving carbonate-C^{14} which is lost as $C^{14}O_2$ during the incubation step.

Conjugates present in the water-soluble metabolite fractions were partially resolved by TLC (Table IV), but no

conjugate was isolated in a pure form. Certain aglycones were tentatively identified after hydrolysis of the various metabolite regions by β-glucosidase (Table V), but the sugar portions of the conjugates were not determined because of their small amount relative to contaminants. As discussed below, the complexity of the water-soluble metabolites may be related to the following: some of the various carbamate metabolites derived from each methylcarbamate are conjugated; in addition to the usual *O*-ether glycosides, other types of glycosides are possibly present, including *O*-enol glycosides and *N*-glycosides; mono-, di-, and trisaccharides possibly serve as the sugar residues in the glycoside formation and different sugars might enter into the reaction.

Each water-soluble metabolite region does not represent a single aglycone conjugated with various sugars, or a single sugar conjugated with various aglycones, because conjugates of compounds such as 4-hydroxy carbaryl, *O*-depropyl Baygon, and *N*-hydroxymethyl Banol appear in each of the partially resolved metabolite regions, and β-glucosidase hydrolyzes, at least partially, conjugates in each separated metabolite region. The conjugates probably consist, for the most part, of *O*-glycosides with carbamate metabolites containing an alcoholic group (*N*-hydroxymethyl carbamates or carbamates having hydroxyl groups in alkyl side chains) or a phenolic group (ring hydroxylated carbamates). They also may contain an *O*-glycoside with the enol form of the carbamate (such as 1-naphthyl methylimidocarbonate *O*-glycoside) or an *N*-glycoside (such as 1-naphthyl methylcarbamate *N*-glycoside), based on analogy with metabolites of carbaryl as recovered from urine (Knaak *et al.*, 1965). Since carbaryl *O*-enol glycoside and *N*-glycoside may be cleaved nonenzymatically or on incubation with β-glucosidase to give carbaryl, it is not surprising that a material similar in chromatographic characteristics to carbaryl is recovered on hydrolysis of several regions of the partially resolved water-soluble metabolites. There is little, if any, evidence that Banol and Baygon are conjugated so that the original carbamate can be recovered by hydrolysis on incubation with β-glucosidase.

Apparently, the sugar residue is more dominant than the aglycone in determining the chromatographic characteristics of the conjugates, which probably contain mono-, di-, and trisaccharides of various sugars. Since it was prepared from almond emulsion, the β-glucosidase used for cleavage probably contains β-glucosidase, β-galactosidase, α-galactosidase, α-mannosidase, and some β-glucuronidase (Pigman and Goepp, 1948; Pridham, 1963). Hence, in addition to the glucosides, the galactosides, mannosides, or glucuronides in the water phase would be expected to be partially cleaved. [β-D-Galactose, β-D-mannose, and β-D-glucuronic acid are known to exist in naturally occurring plant glycosides (Dyke, 1960; Pridham, 1965).] This is a possible explanation for the finding that metabolites in all TLC regions are partially cleaved by β-glucosidase (Table IV). The occurrence of disaccharide- and trisaccharide-containing glycosides in plants is not uncommon (McIlroy, 1951; Pigman and Goepp, 1948; Pridham, 1965). Phenolic glycosides with a disaccharide sugar moiety almost always contain a D-glucose and D-galactose residue, and it is this end of the disaccharide that is bonded by a β-D linkage to the phenol residue (Pridham, 1965) so that cleavage by β-glucosidase or β-galactosidase, respectively, is possible. Thus, regions containing di- and trisaccharide glycosides are also subject to cleavage.

Glusulase, a mixture of β-glucuronidase and sulfatase, cleaves carbaryl water-soluble metabolites to the same or only a slightly greater extent than β-glucosidase (Table III). In another experiment involving various enzymes added in sequence to cleave the carbaryl or Banol water-soluble metabolites, β-glucuronidase and aryl sulfatase separately cleaved only 3 to 6% of additional metabolites over and above the 51 to 53% cleaved by β-glucosidase. With Baygon water-soluble metabolites, the additional cleavage over and above the 43% produced by β-glucosidase was 22% on subsequent addition of β-glucuronidase and 10% on addition of aryl sulfatase (Kuhr, 1966). When the water-soluble metabolites are partially resolved on TLC and then cleaved by β-glucosidase, the extent of cleavage varies greatly with the metabolite region (Table IV). When the experiment is made in the same manner except that β-glucosidase, β-glucuronidase, and aryl sulfatase are added in sequence, the additional cleavage by β-glucuronidase and aryl sulfatase also varies greatly with the metabolite region. Total cleavage of individual resolved water-soluble metabolite regions derived from the three compounds, by the three enzymes (acting in sequence), varies from 21 to 100% (Kuhr, 1966). Water-soluble metabolites not cleaved under these conditions possibly consist of conjugates with sugars or amino acids which are not cleaved by the enzyme preparations used.

The hydroxylation products of methylcarbamate insecticide chemicals as formed in bean plants are similar, in many respects, to those formed in the liver microsome-$NADPH_2$ systems (Dorough and Casida, 1964; Leeling and Casida, 1966; Oonnithan and Casida, 1966). Such products are conjugated as glucuronides and ethereal sulfates in mammals prior to excretion (Knaak *et al.*, 1965; Leeling and Casida, 1966), but on conjugation as glycosides, they are persistent in plants. Whereas a soluble tyrosinase from houseflies hydroxylates phenyl methylcarbamate in the ortho and para position (Metcalf *et al.*, 1966b), mushroom tyrosinase, under cresolase or catecholase assay conditions fails to modify any of the 10 methyl- and dimethylcarbamates used in the present report. Also, horseradish peroxidase is known, under certain conditions, to hydroxylate selected aromatic compounds and oxidatively *N*-demethylate *N,N*-dimethylphenyl compounds (Gillette *et al.*, 1958). But, under the conditions used in the present study, HRP degrades only those carbamates containing a dimethylaminophenyl group (Matacil and Zectran), and the organosoluble products are similar to those formed in the plant and on the surface of the plant (Abdel-Wahab *et al.*, 1966). Water-soluble products formed from these two compounds are carbamates but they are not yet identified. Therefore, plant enzymes other than those studied in this investigation, or the same ones acting under different conditions than those used, must be involved in the *N*-methyl hydroxylation, ring hydroxylation and thioether oxidation that occur in bean plants (Abdel-Wahab *et al.*, 1966).

Many of the aglycones, or hydroxylation products of carbamates formed in the plant, are anticholinesterase agents. Bioassay of several of the aglycones prepared by

synthesis shows that even though they are of high anticholinesterase activity, they are of reduced mammalian toxicity relative to their substituted-phenyl methylcarbamate precursors (Balba, 1967). These aglycones normally exist in the plant as glycosides which probably are less active than the aglycones as cholinesterase inhibitors; some of the products at the origin of the TLC plates which do not show up as inhibitors, possibly represent such unhydrolyzed conjugates. Release of these aglycones from the conjugated form, which may occur on their ingestion by mammals, could result in an additional source of toxic agents. These glycosides of carbamates would go unnoticed among the residues except when determined by a method specifically designed to detect their presence.

In the present study, the methyl- and dimethylcarbamates used were injected into the plants and were not applied under conditions simulating those involved in their actual use as insecticide chemicals. In addition, the studies were restricted to bean seedlings. It is not known whether the same metabolites are formed with other plants or under more practical use conditions, especially where penetration of the precursor compounds into the plant is a necessary preliminary step in their formation.

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