Homeosterically Related Plant Growth Regulators. II. Biological Assay Studies

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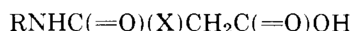
Avena coleoptile segments were grown in the presence of a series of homeosterically related compounds, $RC_6H_4NHCO(X)CH_2COOH$, where (X) = $-OCH_2$(I), $-NHCH_2$(II), $-CH_2CH_2$(III), or -S(IV), and their effects on elongation, as well as protein and carbohydrate composition of the treated segments, were determined. Derivatives in series III decreased the protein content of the treated segments, whereas analogous compounds in series I and II had no significant effect. The unsubstituted phenyl derivatives (R = H) in each of the series did not significantly affect the carbohydrate content of inhibited coleoptile segments; however, the introduction of a chloro substituent (R = Cl) both inhibited segment elongation and decreased the carbohydrate composition as compared to control segments. In subsequent studies on the biological activity of these homeosteres in sunflower seedlings applied *via* foliar application and in hydroponic studies, all were toxic to growth in the latter system, whereas only series III demonstrated growth-inhibitory effects in the former assays.

The plant growth regulatory properties associated with the synthetic herbicide 2,4-D (Zimmerman and Hitchcock, 1942) and/or the substituted phenoxy acids in general (Fawcett *et al.*, 1953) have resulted in the search for structurally related compounds as potential plant growth substances. A variety of different structural compounds have been synthesized, many of which are observed to induce biological responses in plant systems, and these studies have served as the basis for numerous review articles (Wain and Fawcett, 1969). In conjunction with the biological response produced by synthetic plant growth regulators, efforts have been made to correlate these activities with the chemical structures of the compounds (Crozier *et al.*, 1970; Hogue, 1970; Skoog *et al.*, 1967).

Among the variety of chemical structures which have proved to yield compounds with plant growth-regulating properties may be included carbamates (Keitt, 1967), thiolcarbamates (Fang, 1969), ureas (Bruce and Zwar, 1966), and amides (Krewson *et al.*, 1960). It is of interest to note that these four series may be chemically related to each other in a homeosteric manner (Schatz, 1960) by the insertion of methylene groups in a general chemical structure. For example, $RNHCOOCH_2R'$, $RNHCOSR'$, $RNHCONHCH_2R'$, and $RNHCOCH_2CH_2R'$ have mass equivalents of 73, 75, 72, and 71, respectively. Accordingly, four homeosterically related series of plant growth-regulating compounds have been synthesized which are represented by the general structure



$$RNHC(=O)(X)CH_2C(=O)OH$$

where R = hydrogen, cyclohexyl, phenyl, or a variety of mono- and disubstituted phenyl radicals, and (X) = $-OCH_2-$ [series I], $-NHCH_2-$ [series II], $-CH_2CH_2-$ [series III], and -S- [series IV] (Skinner and Sargent, 1973). These derivatives proved to be inhibitory to growth in a variety of plant assay systems, and in the present study an attempt has been made to correlate chemical structure with the biological activity observed using the *Avena* coleoptile assay technique, as well as with whole plant systems utilizing sunflower seedlings and corn plants.

EXPERIMENTAL SECTION

Organic Compounds. The syntheses of the various new compounds utilized in this study are presented in the preceding paper (Skinner and Sargent, 1973).

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Biological Assay Procedures. *Coleoptile Assays.* The procedure utilized for the growth of the coleoptile segments was patterned after the method described by Weigand and Schrank (1959) using Victory variety of *Avena sativa* seeds. Seedlings possessing comparable average lengths were selected and cut (using new sharp razor blades) into uniform 11-mm segments each 5 mm from the tip (Lee, 1963) under a red light source, immediately placed in a 2% sucrose solution, and allowed to equilibrate in the dark at 22° for about 1 hr. Ten randomly selected segments were placed in petri dishes containing 20 ml of the desired concentrations of the test compounds contained in a 2% sucrose solution and the dishes were placed in a high humidity incubator in the dark at 22° for 24 hr. The length of each coleoptile segment was then measured using a small plastic ruler subdivided in millimeters. All assays were repeated a minimum of five times in separate experiments.

Chemical Assay Procedures. As indicated schematically in Figure 1, 20 treated (or control) coleoptile segments were carefully rinsed with distilled water and placed in a glass mortar containing approximately 2 ml of water, and ground by hand for about 2 min. Ten milliliters of water was added, the slurry was sonically disrupted for 1 min, and the homogenate was filtered to remove the debris. The filtrate was brought to a final volume of 20 ml by the addition of water, and a 10-ml aliquot was saved for a dry weight determination. The remaining 10 ml of homogenate was cooled to 5° and treated with 5 ml of cold (5°) 10% trichloroacetic acid. After standing 5 min, the mixture was centrifuged and the supernatant saved for the carbohydrate determination. The residue from the centrifugation was treated with 5 ml of 5% trichloroacetic acid and heated in a water bath at 90–100° for 10 min. The resulting mixture was centrifuged for 3 min. The supernatant solution was decanted and the operation was repeated. The resulting residue was suspended in 10 ml of 0.1 *N* sodium hydroxide and heated at 90–100° for 10 min for use in the protein determination.

Carbohydrate determinations were carried out using the Anthrone method (Dreywood, 1946) at 620 mμ on a Beckman Model DB spectrophotometer. The absorbance readings were converted to μg/ml of carbohydrate by using a Beer's law graph obtained from standard solutions of glucose.

Protein determinations were carried out by the Lowry method (Lowry *et al.*, 1951) using a Beckman Model DB spectrophotometer at 600 mμ. The absorbance readings were converted to μg/ml of protein using Beer's law graph obtained from standard solutions of Beef Serum Albumin.

Table I. Effect of Homeosteres on Elongation of *Avena sativa* Coleoptile Segments[a]

$RNHC(O)(X)CH_2C(O)OH$

	Series							
(X) =	I -OCH₂-		II -NHCH₂-		III -CH₂CH₂-		IV -S-	
			% inhibition[b]					
R group, supplement of IAA, μg/ml	0	1	0	1	0	1	0	1
Phenyl	68	71	15	38	29	49	69	80
2-Chlorophenyl	79	77	12	40	51	61	81	78
2-Chlorophenyl	79	79	29	28	51	66	80	84
4-Chlorophenyl	83	78	28	39	65	73	80	82
2,4-Dichlorophenyl	96	97	84	85	82	84		
2,5-Dichlorophenyl	89	92	10[c]	17[c]				
3,4-Dichlorophenyl	97	93	78	78				
4-Fluorophenyl	70	75	7	21				
4-Bromophenyl	91	84	30	46	70	63		
4-Methyl	72	75	15	36	31	29		
4-Methoxy	68	69	13	21	18	22		
4-Nitro	70	70	30	51	26	44		
Cyclohexyl	53	64	19	42	36	29	81	89

[a] Segments placed in 100 μg/ml concentration of test compounds. Data based on ten segments/treatment and assayed in at least five separate experiments. [b] Based on average elongation of control segments (*e.g.*, 4 mm for no IAA supplement, and 7 mm for 1 μg/ml IAA supplement). [c] A 50 μg/ml solution of this compound was the limit of its solubility.

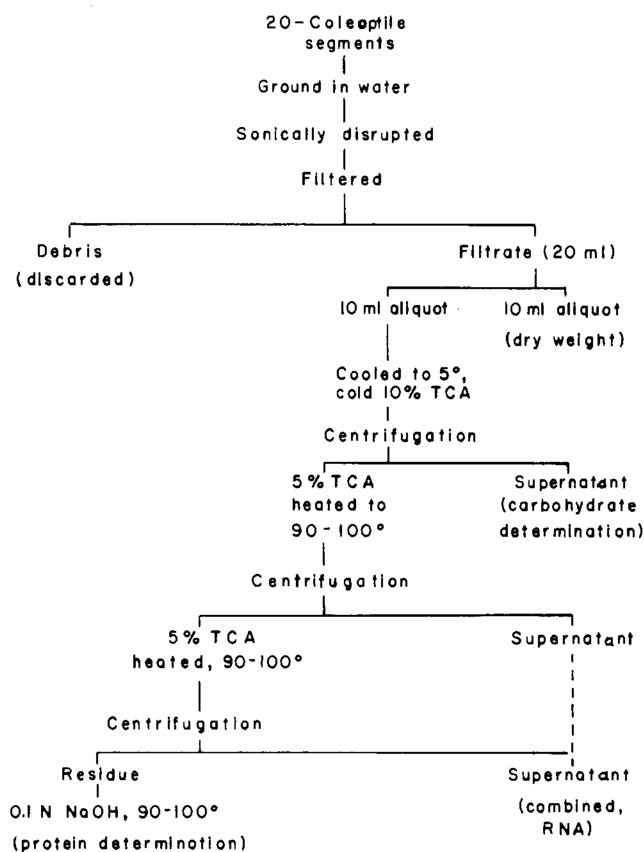

Figure 1. Schematic representation of separation of components for chemical assay.

Sunflower Assays. A *Helianthus annuus* variety of sunflower seeds was utilized to produce the test plants for this study. The plants were grown from seed in an "organic" soil contained in wooden flats. The flats, measuring 8 cm × 23 cm × 36 cm, contained five rows of five seeds each. One row in each flat served as a control (untreated), and the remaining plants were used for two separate bioassays utilizing two consecutive rows. Immediately after planting, the flats were placed in growth hoods approximately 20 cm below a light source consisting of a 500 ft-candle tube (Sylvania Gro-Lux f30T8). The temperature was maintained at 25–27°, and the plants were supplied with tap water as required to maintain adequate growth conditions. Approximately 6–8 days after planting, the flats were adjusted to 30 cm below the light source for the completion of the experiment.

After the test plants reached an average height of approximately 10 cm and/or shortly after the first appearance of the primary leaves (9–11 days from the time of planting), the applications of the test compounds were made. The height was measured to within 0.5 cm for each plant during the experimental period. The test compounds were made up in 1 mg/ml solutions of water which contained 0.1% Tween 20. Using a hand-operated atomizer, the test solutions were applied to the leaves of each plant until the leaves were moist. A second application of the test solution was made approximately 30 min after the leaves had dried. Assuming a 50% vapor loss during spraying, and based on the volume of solution consumed and number of plants involved, the estimated quantity of

Table II. Effect of Homeosteric Modification on Metabolite Composition of Treated *Avena* Coleoptile Segments

$R-C_6H_4-N(H)-C(O)(X)CH_2C(O)OH$

Compound R	(X)	Series	Segment growth	Protein	Carbohydrate
			% inhibition relative to control[a]		
H-	-OCH₂-	I	68	3	0
Cl-	-OCH₂-		83	0	23
H-	-NHCH₂-	II	15	0	+[b]
Cl-	-NHCH₂-		28	0	11
H-	-CH₂CH₂	III	29	16	+[b]
Cl-	-CH₂CH₂		65	13	17

[a] Segments grown in the presence of 100 μg/ml of test compound. [b] A slight increase relative to control was observed.

Table III. Inhibition of Growth of Sunflower Plants by Various Glutaramic Acid Derivatives[a]

$RNHC(O)CH_2CH_2CH_2C(O)OH$

R group	% inhibition[b]
Phenyl	13
o-Chlorophenyl	0
m-Chlorophenyl	62
p-Chlorophenyl	75
2,4-Dichlorophenyl	82
p-Bromophenyl	75
p-Methylphenyl	38
p-Methoxyphenyl	62
p-Nitrophenyl	38
Cyclohexyl	0

[a] Ten-day-old plants were sprayed with a 1 mg/ml solution of the test compounds in a single application until the leaves were moist. [b] Inhibition relative to control untreated plants after 16-day growth period.

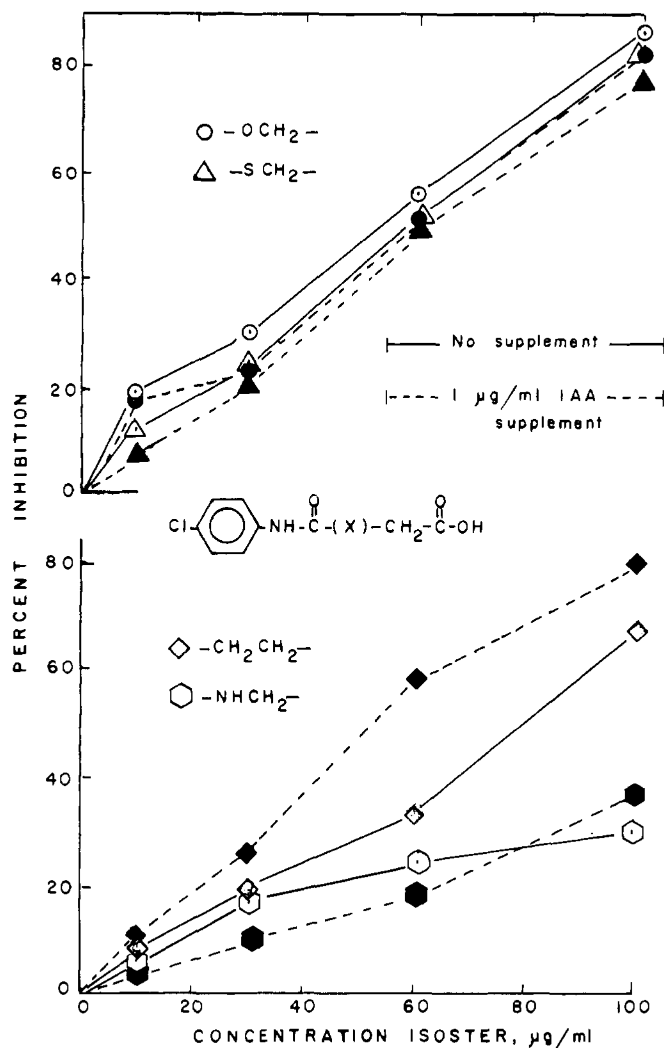

Figure 2. Inhibition of *Avena sativa* coleoptile segment elongation as a function of concentration of homeosteres both alone (clear symbols) and in the presence of auxins (dark symbols).

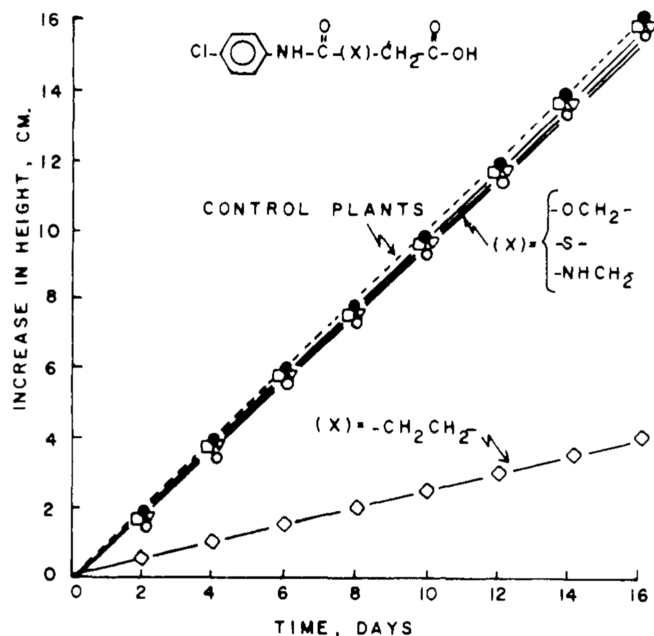

Figure 3. Effect of aerial spray application of homeosteres on rate of growth of sunflower. The solid symbol and dotted line represent a normal growth of untreated plants. Zero time represents 10-day-old plants approximately 10 cm in height.

compound applied to each plant is 0.25 mg. Each treatment was duplicated in at least two separate experiments until consistent and reproducible data were obtained.

Hydroponic Assay. Test plants were grown in moist sand using *Helianthus annuus* sunflower seed and a Golden bantam strain of corn seed. Sunflower plants of approximately 10 cm height (12–14 days) and corn plants approximately 20 cm in height (9–11 days) were then placed in aerated nutrient solution (Mitchell and Livingston, 1968) contained in a 3.5-l. glazed ceramic pot and supported in such a manner that the root system was submerged. The test compounds were added to the nutrient solution to produce a 50 μg/ml of final concentration. The initial height of each plant was determined to the nearest 0.5 cm and measured at 48-hr intervals for a 16-day period.

RESULTS AND DISCUSSION

The physiological growth response on *Avena sativa* coleoptile segments assayed in the presence of the various analogs was determined by a comparison of the average increase in length of the treated segments with that obtained for segments grown in either 2% sucrose alone or 2% sucrose media supplemented with 1 μg/ml of indole-3-acetic acid. For comparative purposes, the complement of the percentage difference in segment elongation was related to the control (*i.e.*, for an average increase in growth of 80% control, the complement is 20%) and recorded as percent inhibition (Table I). These data were determined in the presence of 100 μg/ml of the test compounds and indicate that comparably substituted derivatives *in* the oxygen series (I) and the sulfur series (IV) possess essentially equivalent toxicity to growth. Their inhibitory effects are substantially greater than the analogous homeosteres containing nitrogen and carbon (series II and III). The addition of halogen on the aryl nucleus in each series usually increased the inhibitory activity.

The homeosteric replacement of oxygen by an -NH- group in a series of methylchlorophenylglycines also produced analogs of lesser toxicity than the corresponding oxygen derivatives (Clark and Wain, 1963). Further, replacement of oxygen by sulfur in a series of phenylthioalkanecarboxylic acids produced derivatives of comparable bioactivity in the pea test (Fawcett *et al.*, 1955), whereas in other assay systems, sulfur analogs have been found to be less active, possibly through oxidation to sulfoxide or sulfone structures *in vivo* (Erdtman and Nilsson, 1949).

The urea derivatives (series II) were in general less toxic than the sulfur and oxygen homeosteres to coleoptile elongation, and their growth inhibitory effects were also reversed by supplements of IAA at the lower concentration levels. In contrast, the carbon homeosteres (III) were more toxic to the elongation of the coleoptile segments in the presence of IAA than in the absence of auxin. This latter effect is indicated in Figure 2, wherein studies with a comparable series of derivatives (*i.e.*, the *p*-chlorophenyl analogs) are plotted as a function of inhibitory activity *vs.* concentration both in the presence and absence of supplements of IAA. The presence of auxin did not significantly alter the toxicity in either the sulfur or oxygen series and was somewhat inconsistent in the nitrogen series. However, IAA clearly augments the toxicity of the carbon homeosteres (series III), suggesting that these derivatives may have a different mode of action. It should be noted that the carbon homeosteres do not have comparable unshared electrons for bonding as do the other moieties.

A possible explanation for these differential toxicities may be related to the hydrophilic–lipophilic character of the compounds. The introduction of halogens (usually chloro or bromo) into the benzene ring of an analog often produces a more favorable hydrophilic–lipophilic balance

Table IV. Qualitative Response on Plant Growth of Homeosteres Assayed under Hydroponic Conditions[a]

RNHC(O)(X)CH$_2$C(O)OH[b]

(X) =	-OCH$_2$- Series I		-NHCH$_2$- Series II		-CH$_2$CH$_2$- Series III		-S- Series IV	
Inhibitory response[c]	Corn	Sunflower	Corn	Sunflower	Corn	Sunflower	Corn	Sunflower
Plant height	++	+++	+	+	++	+++	−	++
Total root system	++	+++	+	+	+++	+++	++	+++
Secondary and lateral root system	+++	+++	++	++	+++	+++	+++	+++
Aerial system	+	++++	−	+	++	+++	−	+++

[a] Plants grown in hydroponic media containing 50 μg/ml of test compounds; response compared to plants grown in hydroponic media alone. [b] R = phenyl, substituted phenyl, cycloalkyl. [c] − = no effect relative to untreated control plants; + = obvious visual response.

and subsequently increases the biological activity of plant growth regulators (van Overbeek, 1959).

An alternate explanation for the differences in biological activity of these compounds may involve their ability to bind at or near active enzymic sites and thus effect an inhibition of essential metabolic processes. The presence of a number of potential binding sites in the four series described may permit them to form electrostatic attachment on an enzyme surface (Barth, 1969), or exert their effect through van der Waals forces (London, 1930) or through interaction with a combination of enzymic sites (van Overbeek, 1961).

In an effort to determine if the growth inhibitory responses are associated with metabolic effects resulting in changes in cellular composition, both treated and control coleoptile segments were assayed for protein and carbohydrate content using the *p*-chloro- and the corresponding unsubstituted phenyl homeosteres in series I, II, and III as model compounds.

As indicated in Table II, neither derivative in the oxygen or nitrogen series (I or II) affected the protein composition, whereas both the phenyl and *p*-chlorophenyl analogs in series III produced a decrease in the protein content of the segments relative to the controls. The unsubstituted phenyl homeosteres in series I, II, and III did not reduce carbohydrate content relative to control segments; however, segments grown in the presence of the *p*-chlorophenyl analogs did produce a decrease in carbohydrate composition. Of interest is the fact that carbohydrate production was actually increased in coleoptiles grown in the presence of unsubstituted phenyl analogs of series II and III. It would appear that the introduction of electronegative substituent is essential for maximum toxicity, and an inhibition of carbohydrate biosynthesis by these derivatives may well explain the greater inhibitory activity of the *p*-chlorophenyl analogs.

In view of the growth-regulating activity of these homeosteres in coleoptile assays, it was of interest to carry out a preliminary study of their biological activities in whole plant systems. Aerial spray application of the *p*-chlorophenyl derivative in each of the four series early indicated that *N*-(*p*-chlorophenyl)glutaramic acid (series III) was the most inhibitory to growth (Figure 3). Subsequently, a study of the effect of various substituent groupings on the aromatic nucleus in series III demonstrated that derivatives with a para- or meta-halo substituent were the most effective in reducing the rate of growth of sunflower seedlings (Table III). For example, the *o*-chlorophenyl compound is inactive in inhibiting the rate of growth of sunflower seedlings, whereas both the *m*- and *p*-chlorophenyl derivatives are toxic. There are numerous instances in studies of plant growth regulators where ortho substitution has been demonstrated to negate or reduce biological activity (Barth, 1969; Hansch and Muir, 1950). It is interesting to compare these results with those obtained in the *Avena* coleoptile assay system. In the latter study, comparable activities were obtained for the phenyl, *p*-methylphenyl, *p*-methoxyphenyl, and *p*-nitrophenyl derivatives, whereas in whole growth studies, the substituted phenyl derivatives are uniformly more active than the unsubstituted phenyl analog.

These growth retardation effects may be of potential commercial interest; *e.g.*, foliar application might retard growth (even without killing) of noxious weeds and permit normal growth of the desired crop. The lack of inhibitory activity of certain analogs might be due to an inability to permeate the leaf surface because of selective absorption characteristics (Crafts and Yamaguchi, 1960; Franke, 1967), and since root systems are less selective, it became of interest to study these homeosteres in hydroponic assay systems. In hydroponic assays, the derivatives were more inhibitory to the growth of sunflower than to the commercially important corn plant. Homeosteres containing an oxygen or methylene linkage (series I and III) produced comparable inhibitory effects which were generally more apparent than those produced by analogs in the nitrogen and sulfur homeosteres (series II and IV). The urea derivatives were uniformly less toxic to growth than the other three homeosteric series (Table IV).

All of the homeosteres applied *via* hydroponic techniques did inhibit, to some degree, sunflower growth. Thus, it would appear that the lack of inhibitory activity through spray application at comparable concentration level in the soil-growth studies may be due to permeability phenomena associated with the leaf structure.

ACKNOWLEDGMENT

The authors wish to express their appreciation for support through a National Science Foundation grant no. GB-20512 and Robert A. Welch Foundation (Grant No. B-342).

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Received for review March 19, 1973. Accepted August 8, 1973.

Syntheses of the β-D-Glucosides of 4- and 5-Hydroxy-1-naphthyl *N*-Methylcarbamate

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4- and 5-(*N*-Methylcarbamoyloxy)-1-naphthyl tetra-*O*-acetyl-β-D-glucopyranoside were synthesized by the condensation of the appropriate hydroxy-1-naphthyl *N*-methylcarbamate with β-D(+)-glucose pentaacetate in the presence of catalytic amounts of boron trifluoride–ether complex. Reaction of the acetylated β-D-glucosides with barium methoxide in methanol gave the corresponding β-D-glucosides. The ir, nmr, and mass spectra of the β-D-glucosides and their acetylated analogs are reported and compared. When administered ip to mice, 4-hydroxyl-1-naphthyl *N*-methylcarbamate was 28 times more toxic than its β-D-glucoside and 5-hydroxy-1-naphthyl *N*-methylcarbamate was 19 times more toxic than its β-D-glucoside. The methyl ester of the β-D-glucuronide of 5-hydroxy-1-naphthyl *N*-methylcarbamate was prepared in a manner similar to the glucosides. However, attempts to demethylate the product while leaving the carbamate ester intact were unsuccessful. Attempts to synthesize the glucoside of the 3-hydroxy derivative of carbofuran also were unsuccessful.

The metabolism of carbaryl (Sevin, 1-naphthyl *N*-methylcarbamate) in plants has been intensively investigated (Abdel-Wahab *et al.*, 1966; Dorough and Casida, 1964; Dorough and Wiggins, 1969; Kuhr and Casida, 1967; Mumma *et al.*, 1971). A review of the pathways of carbaryl and other methylcarbamate insecticide metabolism in plants has been recently reported (Kuhr, 1970). The predominant pathway involves oxidative metabolism, while hydrolysis usually occurs to a relatively small extent.

Oxidative metabolites of carbaryl which result from hydroxylation of the ring and the *N*-methyl group are not recovered as such, but are rapidly converted into stable water-soluble products by conjugation, mainly as glycosides (Kuhr and Casida, 1967; Mumma *et al.*, 1971). The major aglycones are 4-hydroxy-1-naphthyl *N*-methylcarbamate (4-hydroxycarbaryl), 5-hydroxy-1-naphthyl *N*-methylcarbamate (5-hydroxycarbaryl), 1-naphthyl *N*-hydroxymethylcarbamate (*N*-hydroxymethylcarbaryl), and *trans*-5,6-dihydro-5,6-dihydroxy-1-naphthyl *N*-methylcarbamate. While these metabolites also are present in mammals and insects they are readily eliminated, whereas plants store them as conjugates for a considerable length of time (Kuhr, 1970).

Although it has been reported that the water-soluble metabolites of carbaryl in bean plants were readily eliminated from the rat (Dorough and Wiggins, 1969), the metabolism and toxicological properties of carbaryl conjugate metabolites have not been critically evaluated. Since the aglycones possess anticholinesterase activity (Dorough, 1970), and their release from the conjugated form could occur in mammalian systems, the need for further study of these compounds is apparent. Such a study would be greatly facilitated by the chemical syntheses of the intact conjugate metabolites. This has been accomplished with certain carbaryl metabolites in the current investigation.

Chemical syntheses and acute toxicity of the β-D-glucosides of carbaryl metabolites, 4-(*N*-methylcarbamoyloxy)-1-naphthyl β-D-glucopyranoside and 5-(*N*-methylcarbamoyloxy)-1-naphthyl β-D-glucopyranoside, are reported. The preparation of their respective decarbamylated products, 4-hydroxy-1-naphthyl β-D-glucopyranoside and 5-hydroxy-1-naphthyl β-D-glucopyranoside, is also reported. These syntheses can provide material for toxicological evaluation and will enable identification of the intact plant conjugates without resorting to hydrolysis of the glycones. The synthetic conjugates may serve as standards to aid in the determination of the sugar moiety of carbaryl plant conjugates, an important consideration which up to this time has been lacking.

MATERIALS AND METHODS

Chemicals. 1,4-Naphthalenediol and 1,5-naphthalenediol were purchased from Eastman Kodak Co. 4- and 5-Hydroxycarbaryl were synthesized by the reaction of the corresponding naphthalenediol with methyl isocyanate (Knaak *et al.*, 1965). β-D(+)-Glucose pentaacetate, α-glucosidase, and β-glucosidase were purchased from Sigma Chemical Co., and boron trifluoride–ether complex (98%) was purchased from Matheson Coleman and Bell. Methyl tetra-*O*-acetyl-β-D-glucopyranuronate was synthesized by

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