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Title: A COMPARISON OF THE RATES AND PATHS OF METABOLISM OF 2, 4-DICHLOROPHENOXYACETIC ACID IN RESISTANT AND SUSCEPTIBLE PLANTS

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Abstract approved:

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Studies on the absorption of C^{14} carboxyl labeled 2, 4-dichlorophenoxyacetic acid by a susceptible plant (bean) and and resistant plants (corn and bluegrass) indicated that the chemical was readily absorbed by all species of plants from a foliar application. Approximately 22% and 42% of the applied chemical was absorbed by bean and corn plants respectively in three days following application of a 250 ppm solution of herbicide. There were no significant differences in rates of absorption of 2, 4-D in extended exposure. Approximately 60% and 65% of 2, 4-D solution were absorbed by either bean or corn plants in seven and 11 days respectively.

In metabolic studies, corn plants were found to conjugate the C^{14} carboxyl labeled 2, 4-D much more rapidly than the susceptible bean plants. Analysis of the plant extract by paper chromatography

revealed that corn had metabolized all of the absorbed chemicals in three days following application, while the beans had metabolized approximately 60% of the absorbed 2, 4-D. At the end of 11 days, the bean plants had metabolized approximately 88% of the herbicide to a conjugate.

Enzymatic hydrolysis followed by paper and thin-layer chromatography of the plant extracts indicated that the metabolites of 2, 4-D were present in the plants as glucose adducts. Two metabolites beside the parent compound 2, 4-D itself were observed in both types of plants following hydrolysis by either acid, base, or enzyme.

The metabolites were identified as 2, 5- and 2, 3-dichloro-4-hydroxyphenoxyacetic acid by means of R_f values in thin-layer chromatography and retention times in gas chromatography. Hence, the pathways of 2, 4-D metabolism in bean, corn, and bluegrass plants would seem to be through hydroxylation of the ring of phenoxy herbicide accompanied by a chlorine shift. Determination of the areas under the chromatographic peaks of these two metabolites indicated that they were present in a ratio of approximately 3:1, 6:1, and 10:1 respectively in 3, 7, and 11 days following treatment.

The evolution of $C^{14}O_2$ by plants revealed that this was a minor pathway of detoxification and there were no significant differences in rates of decarboxylation between susceptible bean and resistant corn plants. Furthermore, no significant amounts of unextracted

radioactivity were found to be accumulated in plants.

The foregoing evidence indicates the routes of metabolism of plants are through decarboxylation, conjugation, and hydroxylation accompanied by a chlorine shift. The ability of plants to conjugate 2, 4-D and its metabolites as glucose adducts becomes the major point of difference between the susceptible and resistant plants.

A Comparison of the Rates and Paths of Metabolism of 2, 4-Dichlorophenoxyacetic Acid in Resistant and Susceptible Plants

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INTRODUCTION

2, 4-dichlorophenoxyacetic acid (2, 4-D) was first synthesized by Pokorny (1941). Its growth regulating properties were subsequently discovered by Zimmerman and Hitchcock (1942). The application of this compound of high biological activity to kill plants was first described by Marth and Mitchell (1944). They found that numerous broad-leaved weeds in lawns were destroyed by relatively small amounts of chemical. Since that time millions of pounds of 2, 4-D has been used as a herbicide on crops. The success with this compound stimulated an extensive search for additional chemicals for use as herbicides.

The extensive use of chemicals to kill plants required development of understanding of the mechanism of selectivity of herbicides. Biochemical selectivity has been received the primary interest, although physical selectivity is frequently encountered. Where physical selectivity is involved, the chemical is applied in such a manner that the crop plants receive a minimum exposure to the chemical.

In the case of biochemical selectivity, one of two major factors is operative. First, the biochemical systems of the tolerant plant may be unaffected by the presence of the herbicide. Such selectivity has been demonstrated with the herbicide 2, 2-dichloropropionic acid (Smith and Dyer, 1961) and trichloroacetic acid (Andersen et al.,

1962; Blanchard, 1954). This type of selectivity has not been encountered very frequently.

The second type of biochemical selectivity is resistance through detoxication. The herbicide is metabolized by the tolerant plant to innocuous or less toxic metabolites before it can exert its toxic action. This type of selectivity is the one which has been most frequently encountered.

From a broader point of view, metabolism studies provide not only significant information on the path of degradation and basis of selectivity, but also gives insight into the mode of action and probable residue hazards as well. The persistance of these chemicals and their metabolic products in plants must be known to insure maximum safety to crop plants as well as to man.

Although the metabolic fate of 2, 4-D in plants has been widely and extensively studied, the details of its metabolism remain unclear. There is no agreement on the extent, rate, or pathway of degradation of this chemical. In addition, most of the investigators have worked with plants sensitive to the chemical, thus leaving in doubt whether or not the tolerant plants such as grasses are actually capable of absorption and translocation of the chemical or whether the tolerance is due in part to the plant ability to metabolize the chemical (Freed and Montgomery, 1963).

The purpose of this study was to compare the rates and paths of

metabolism of 2, 4-D in resistant and susceptible plants. This study would provide the information as to the actual mechanism of detoxication of 2, 4-D in resistant plants.

REVIEW OF LITERATURE

A number of studies on the metabolic fate of herbicides in plants show that most herbicides are metabolized in varying degree. Shaw et al. (1960) indicated that species may differ greatly in either: (a) their ability to metabolize a specific herbicide or, (b) the biochemical pathways by which the structure of the original molecule is altered. For some chemicals, this difference seems to be the determining factor in herbicidal selectivity.

Considerable evidence indicates that 2, 4-dichlorophenoxyacetic acid (2, 4-D) is metabolized by plants. Paths of metabolism of 2, 4-D include (a) conjugation of 2, 4-D or its metabolites with plant constituents, (b) alterations in the structure of the parent molecule, and (c) partial degradation with loss of the acetic acid side chain. However, a relatively small percentage of unaltered 2, 4-D may be recovered from metabolically active plants.

The various types of metabolism of herbicides in plants represent either activation or detoxification mechanisms. It is therefore not known whether 2, 4-D or one of its metabolized forms is the toxic principle responsible for the major biological and biochemical changes observed in plants treated with 2, 4-D (Shaw et al., 1960). Edgerton and Hoffman (1961), and Luckwill and Lloyd Jones (1960a, b) demonstrated that there is a good correlation between the resistance of a

plant to a chemical and the plant's ability to metabolize it. Therefore, it is very likely that 2, 4-D itself is responsible for the toxication in plants, while the metabolism of this chemical is responsible for detoxification.

Conjugation with plant constituents of the parent herbicide as well as its metabolites has been found as one of the primary metabolic processes. Klämbt (1961) demonstrated the formation of glucose ester of 2, 4-D in wheat coleoptiles. The work of Jaworski and Butts (1952) suggested the same product was formed in bean tissue. Thomas et al. (1964a, b) further indicated the accumulation of the glucose ester of 2, 4-D as 1-0-(2, 4-dichlorophenoxyacetyl)-β-D-glucose in bean tissues is a major metabolic pathway of 2, 4-D in plants.

Other 2, 4-D complexes are formed with amino acids and proteins. Andreae and Good (1957) first proposed the formation of an amino acid adduct in pea epicotyls. Klämbt et al. (1961) demonstrated the metabolic formation of 2, 4-D aspartic acid in wheat coleoptile cylinders. Bach and Fellig (1961) found ten ninhydrin-positive metabolites of 2, 4-D in bean plants, and indicated that conjugation was rather non-specific.

Protein complexes of 2, 4-D have been found in a number of plants by Fang (1958), and Butts and Fang (1956). These 2, 4-D-protein complexes were found in pea and tomato plants (Fang, 1958) as well as in corn and wheat (Butts and Fang, 1956). Hydrolysis of

these protein complexes produced aspartic acid, glutamic acid, glycine, serine, threonine, alanine, tyrosine, methionine, phenylalanine, valine, leucine, and isoleucine. A comparable amount of the same amino acids were produced after hydrolysis of the major metabolites of 2, 4-D-protein complexes, which suggested a similar origin. The parent compound can be released intact by hydrolysis of these complexes. The conjugation of 2, 4-D with protein followed by extensive degradation of an unspecific nature was believed to be the major metabolic pathway of the herbicide (Canny and Markus, 1960); whereas such 2, 4-D complexes were proposed as a minor pathway (Leafe, 1962).

Other 2, 4-D complexes have been shown, but the nature of the products has not been investigated. About half of the 2, 4-D absorbed by bean was bound in the tissue, and was hydrolyzable by 2N hydrochloric acid (Bach and Fellig, 1959a, b). Further evidence for a bound form of 2, 4-D has been presented by Luckwill and Lloyd-Jones (1960a,b). These authors showed that as much as 30% of the 2, 4-D applied was found as a conjugate in currants as well as in apple and strawberry leaves. In these cases, weak hydrolytic conditions would not release free 2, 4-D. Others have indicated the presence of unidentified and uncharacterized complexes (Waite Agricultural Research Institute, 1959). McCready (1963) surmised that a labile complex acted as the translocation form of 2, 4-D.

In addition to conjugation, 2, 4-D undergoes extensive oxidation. Metabolism through oxidation ranges from simple hydroxylation to complete oxidation of the molecule to carbon dioxide and other products. The initial 2, 4-D metabolism were studied by Holley et al. (1950) and later by Holley (1952). These investigations indicated that 2, 4-D was oxidized by bean plants to a more polar compound which appeared to be an aryl hydroxylated product. The metabolic product was conjugated with plant constituents. This finding was confirmed by Thomas et al. (1964b) who found beans formed two conjugated phenolic acids from 2, 4-D. The primary product was 4-OH-2, 5-D (4-hydroxy-2, 5-dichlorophenoxyacetic acid). A small amount of 4-OH-2, 3-D (4-hydroxy-2, 3-dichlorophenoxyacetic acid) was also formed. This suggested that hydroxylation as well as a novel chlorine shift had taken place. The chlorine shift was not unique to plants since 4-OH-2, 5-D was shown to be a minor metabolite of 2, 4-D in Aspergillus niger (Faulkner and Woodcock, 1965). However, the major fungal metabolite of 2, 4-D was 5-OH-2, 4-D.

As mentioned above, 2, 4-D undergoes decarboxylation as well as hydroxylation in the oxidative reaction of the chemical. The evolution of $C^{14}O_2$ from a number of plants exposed to either methylene or carboxyl labeled 2, 4-D has been repeatedly demonstrated (Holley, 1950; Weintraub et al., 1950, 1952, 1954). Weintraub et al. (1950) found that the plants evolved $C^{14}O_2$ three

times more rapidly from the carboxyl than from the methylene position. No measurable amount of $C^{14}O_2$ was produced from plants treated with ring labeled 2, 4-D (Weintraub et al., 1952). It has been suggested by Weintraub et al. (1950) that the two carbon side chain was liberated and subsequently entered the tricarboxylic acid cycle prior to decarboxylation. Weintraub et al. (1956) further demonstrated that there was no marked difference in decarboxylation of 2, 4-D between bean and corn plants. Both species were found to produce a relatively little $C^{14}O_2$. Therefore, Weintraub et al. suggested that the resistance of plants to 2, 4-D was probably not associated with rapidity of decarboxylation of the herbicide. The work of Bach and Fellig (1959a,1961) supported the above suggestion.

On the other hand, others have found marked differences in species and varietal ability to decarboxylate 2, 4-D. Thus, resistant McIntosh apples decarboxylated much more 2, 4-D than the susceptible Winesap and Stayman varieties (Edgerton, 1961; Edgerton and Hoffman, 1961). Luckwill and Lloyd-Jones (1960a, b) further confirmed the work of Edgerton. They found that there was a good correlation between the degree of decarboxylation and the relative resistance of varieties of currants, apples, and strawberries to 2, 4-D.

The foregoing literature seems to indicate that the metabolism of 2, 4-D includes conjugation, hydroxylation as well as chlorine shift, and decarboxylation. Different metabolites may be formed in

different plant species. Also, while a given metabolic pathway may predominate, it is probable that two or more of the detoxification mechanisms may be operative simultaneously in the same plant. It is anticipated that more detoxification mechanisms may be discovered as further studies are carried out.

MATERIALS AND METHODS

C^{14} carboxyl labeled 2, 4-D (2, 4-dichlorophenoxyacetic acid) was used for most of the experiments concerned with metabolism. However, non-radioactive 2, 4-D was used to characterize the metabolites found in the studies of metabolism. The study was therefore divided into two major parts. The first part was conducted to determine the rates and paths of metabolism of C^{14} 2, 4-D in bean (susceptible plant) and corn (resistant plant). The second part of the study was designed to further characterize the metabolites of non-radioactive 2, 4-D in plants.

C^{14} Carboxyl Labeled 2, 4-D Experiments

Bean plants (Phaseolus vulgaris, variety Top Crop), corn plants (Zea mays, variety Tendermost), and bluegrass plants (Pos protensis L., variety Newport) were used in these studies. Four seeds each of the appropriate plants were germinated and grown in soil in cans. Three cans each of bean plants and corn plants were used in individual experiments. Plants were cultured under general greenhouse conditions until attaining proper development for treatment. Each plant was treated with 0.1 ml of solution containing 250 ppm C^{14} carboxyl labeled 2, 4-D (3.03 millicuries per millimole) as triethanol amine salt, and 0.1% surfactant Tween 20 to enhance

foliar penetration.

The foliar application of 2, 4-D was chosen for two reasons. First, exposure of the plants through the roots resulted in very poor translocation of the chemical from roots to the tops of the plants. Hence, insufficient concentration of chemical in the tops of the plants limited the study of metabolism in the tops. Secondly, application of herbicide to the soil would result in degradation of the chemical by soil microorganisms. The resulting metabolites may be absorbed by the plants and make it impossible to differentiate between those formed by plant metabolism and those resulting from microbial degradation of 2, 4-D.

Four plants of each kind were harvested at the time intervals of three days, seven days, and 11 days following application of 2, 4-D solution. The plants were excised at a height of approximately one inch above the soil. The unabsorbed 2, 4-D which remained on the plants was removed by rinsing the treated leaves with 1% sodium bicarbonate solution.

The plant tissues were extracted in a waring blender with 80% ethanol. The plant macerates were then heated on the steam bath for three hours and filtered. Plant residues were then resuspended in 80% ethanol and extracted again as before. The alcohol solutions were combined and concentrated on a flash evaporator to a volume of 50 ml. Approximately 99% of the absorbed radioactivity was

recovered in the 80% alcohol extract by this method. Ethanol was used as the extracting solvent since it has been shown to be a good solvent for extraction of herbicides and metabolites from plant tissues (Thomas et al., 1964b).

The amount of radioactivity in both the extracts and extracted tissues was determined with a Tracerlab Versamatic II thin window (0.9 mg/cm^2), gas flow Geiger-Muller counter. The efficiency of this instrument is approximately 10% for C^{14}. Aliquots of the extracts were spotted in one inch, concentric-ringed, stainless steel planchets for counting. The C^{14} in the extracted tissues was determined by counting a 10 mg sample of finely ground tissues in a flat-bottomed planchet. Correction was made for self absorption of radioactivity by plant tissues.

Paper chromatography was used to determine the extent of metabolism of the absorbed 2,4-D in plants as evidenced by the appearance of additional radioactive spots. An aliquot of each extract from various harvest times was spotted on strips of Whatman No. 1 paper and developed in acid developer, benzene: acetic acid: water (2:2:1) and alkaline developer, butanol saturated with 1.5 M ammonium hydroxide (BNW). Radioactive areas were located by scanning paper strip with a Vanguard windowless, gas flow scanner. The relative amount of each radioactive area was obtained by determining the areas under the chromatographic peaks from the recorded scan.

The formation of a complex of 2, 4-D with glucose, amino acids, or proteins by conjugation has been demonstrated by Jaworski and Butts (1952), Thomas et al. (1964a,b), Bach (1961a, b), Fang (1958), and Butts and Fang (1956). The 2, 4-D and its metabolites have been found to be released by hydrolysis. To determine whether 2, 4-D was present as a sugar or peptide conjugate, enzymatic hydrolysis as well as chemical hydrolysis of the 2, 4-D complexes was carried out in this study.

The extracts of plants (bean, corn, and bluegrass) were partially purified by paper chromatography and developed in butanol saturated with 1.5 M ammonium hydroxide (BNW) solvent system. Free 2, 4-D which was not metabolized by plants was removed, and the partially purified complexes of 2, 4-D were recovered by extracting the paper for 24 hours in a soxhlet apparatus with 80% ethanol. Aliquots of the alcohol extracts were evaporated to dryness under vacuum. The residues were dissolved in 8 ml buffer (acetate buffer, 0.4 M, pH 5, and tris buffer, 0.01 M, pH 7.2). Four ml of each solution was used as control, another 4 ml was used for enzymic hydrolysis. One ml of each one of the two buffers and each one of the two enzymes (β-glucosidase, 2 mg/ml, Lot 123 B-0330, from almonds, Sigma Chemical Company, and pronase, 1 mg/ml, Lot 53177, β-grade, from Calbiochem.) was added separately to make a 5 ml final volume. The solutions were incubated in a water bath at

37°C with constant shaking for 16 hours. Enzymic reaction was stopped by adding 1 ml of 6M HCl at the end of incubation period. The acid solution was then extracted three times with ether to remove 2, 4-D and its metabolites. The ether solution was concentrated to a small volume under a stream of nitrogen. The extent of hydrolysis was determined by thin-layer chromatography (on silica gel-G plates of 250 micron thickness) of the ether extracts with petroleum ether: ether:formic acid (50:50:2) as developer.

The chemical hydrolysis was accomplished by using either acid or base to release the 2, 4-D and its metabolites from the complexes. Accordingly, the residues of partially purified conjugates of 2, 4-D were dissolved in water, and subjected to either acid (hydrochloric acid) or base (sodium hydroxide) hydrolysis. The final concentration of either acid or base of the hydrolysates was brought to one molar. The solutions were then incubated in water bath at 37°C for 16 hours. The basic hydrolysate was adjusted to pH 1 with hydrochloric acid prior to extraction. The solution was then extracted with ether to remove the 2, 4-D and its metabolite. The ether extracts were concentrated to a small volume and chromatographed on thin-layer silica gel-G plates.

Three radioactive compounds were detected on thin-layer chromatograms. The proportion of these metabolites was determined by comparison of the areas under the chromatographic peaks of each metabolite. In order to identify these compounds, it was necessary to synthesize the various possible hydroxylated products

of 2, 4-D so that their R_f values could be determined. These compounds were prepared by published methods.

6-hydroxy-2, 4-dichlorophenoxyacetic acid (6-OH-2, 4-D). This compound was prepared by condensation of 3, 5-dichlorocatechol (Dakin, 1909) with n-butyl chloroacetate according to the method described by Cavill and Ford (1954). After two recrystallizations from water, the product melted at 134° C. Above authors reported the melting point to be 132° C.

5-hydroxy-2, 4-dichlorophenoxyacetic acid (5-OH-2, 4-D). 5-OH-2, 4-D was prepared by condensation of 4, 6-dichlororesorcinol with chloroacetic acid according to the method described by Moszaw and Wojciechowski (1954). The melting point of this compound was 175.5° C.

3-hydroxy-2, 4-dichlorophenoxyacetic acid (3-OH-2, 4-D). The method described by Faulkner and Woodcock (1965) was used to prepare this compound. 3-OH-2, 4-D was synthesized by condensation of 2, 4-dichloro-3-nitrophenol (Groves et al., 1929) with bromoacetate followed by reduction and diazotization of the 3-amino-2, 4-dichlorophenoxyacetate with sodium nitrite in sulfuric acid. 3-OH-2, 4-D was obtained upon adding the diazonium solution to a mixture of sulfuric acid and water. The melting point of this compound was 172° C.

4-hydroxy-2, 5-dichlorophenoxyacetic acid (4-OH-2, 5-D). This compound was prepared by condensation of 2, 5-dichlorohydroquinone

(Ling, 1892) with ethyl bromoacetate according to the method described by Faulkner and Woodcock (1965). After two recrystallizations from water, the chemical melted at 164° C.

4-hydroxy-2, 3-dichlorophenoxyacetic acid (4-OH-2, 3D). Essentially the same preparation as that described by Faulkner and Woodcock (1965) was used for the synthesis of 4-OH-2, 3-D. 4-OH-2, 3-D was prepared by reacting of 2, 3-dichlorohydroquinone (Wheeler and Liddle, 1923) with ethyl bromoacetate. The melting point of this compound was 168° C.

The R_f values of non-labeled hydroxy-analogs of 2, 4-D were determined by spraying with 1% p-nitrobenzendiazonium fluoborate in 50% acetone solution. The compounds yielded yellow to brown spots with this developing reagent. At least 10 ug of each compound was required for detection.

In order to establish that the same metabolic products of 2, 4-D were present in both susceptible and resistant plants, the metabolites in the extracts of both plants were identified by means of retention times in gas liquid chromatography. The extracts following hydrolysis were further purified by thin-layer chromatography. The radioactive areas were located by a Vanguard chromatogram scanner, and extracted in a soxhlet extractor with distilled acetone for 24 hours.

The acetone extracts were evaporated to dryness under vacuum in a flash evaporator. The residues were then separately treated with ethereal diazomethane or diazo-n-propane to give methyl and propyl ethers and esters respectively. The authentic compounds were esterified in the similar manner for comparison.

After concentration of the ether solution to a small volume, the esters were injected into a Dohrman microcoulometric gas chromatograph to determine their retention times. A five foot, quarter inch column packed with 7% OV-1 on 60/80 gas chrom Q was used for the separation. This instrument combusts the effluent of a gas chromatographic column, and titrates the chlorine liberated from chlorinated compounds. Approximately 50 nanograms of either 2, 4-D or its hydroxy-analogs were necessary for detection.

Diazomethane was prepared according to the method described by Aldrich Chemical Company, Inc., while diazo-n-propane was prepared according to the method described by Dyer et al. (1964).

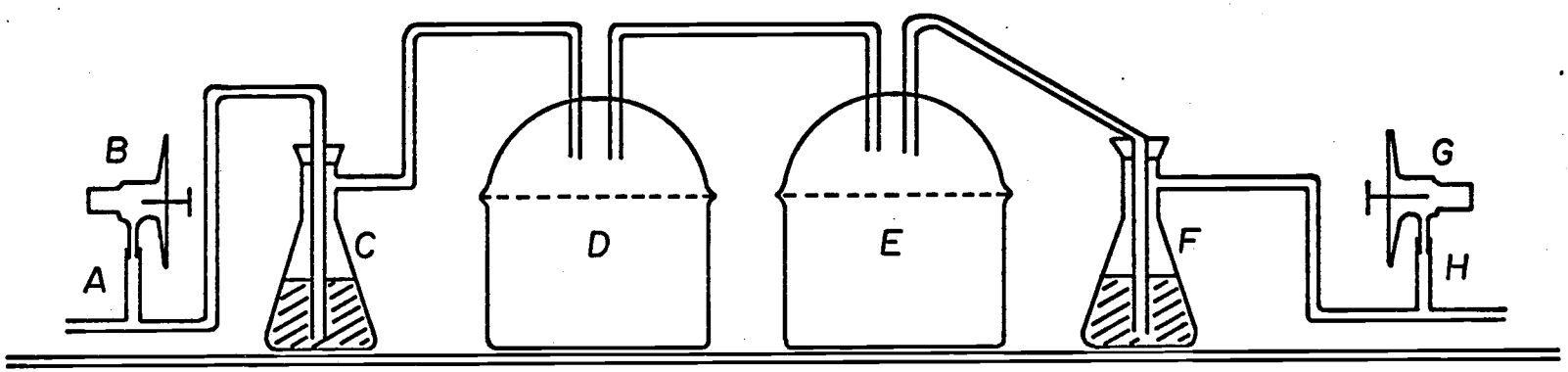
The above studies were designed to determine the nature of the C^{14} metabolites arising from C^{14} carboxyl labeled 2, 4-D. However, if decarboxylationwere occurring, there would be metabolites which would not be detected by radiometric methods of analysis. The decarboxylation studies were therefore separately carried out to investigate the possibility of evolution of $C^{14}O_2$ from both susceptible and resistant plants.

Eight bean plants and twelve corn plants were used in these studies. Each plant was treated with 100 microliters of solution containing 200 ppm C^{14} carboxyl labeled 2, 4-D (12. 1 millicuries per millimole) as the triethanol amine salt and 0. 1% surfactant Tween 20 to enhance penetration from a foliar application.

Four bean plants (or six corn plants) per jar were placed in a three liter desicator modified to serve as a respiratory chamber (see Figure 1). This was done in duplicate for each plant specie. The chambers were swept with CO_2 free air. The outgoing air from the chambers was scrubbed in one molar carbonate free sodium hydroxide.

The decarboxylation studies were run for 48 hours. At the end of the experiment, the chambers were opened, and the surfaces of leaves of the treated plants were rinsed with 1% sodium bicarbonate solution, and then with distilled water to remove unabsorbed chemical. The plants were extracted with 80% ethanol to determine the amount of C^{14} that was absorbed by the plants.

The one molar sodium hydroxide carbon dioxide trapping solution in the outgoing air scrubbing towers was quantitatively transferred into erlenmeyer flasks and the carbonate ions precipitated as barium carbonate with one molar barium chloride. Aliquots of the barium carbonate solutions were filtered through previously weighed 2. 1 centimeter spun-glass filter discs. The filtered carbonates were



A. Air line
B. Needle valve
C. CO_2 trap for incoming air
D,E. Respiratory chamber
F. CO_2 trap for outgoing air
G. Needle valve
H. Vacuum line

Figure 1. Respirator

washed with water and methanol; and air was drawn through until the carbonate was dry. The carbonate plates were reweighed and counted on one inch flat bottomed, stainless steel planchets with a Tracerlab Versamatic II thin window gas flow Geiger-Muller counter. Suitable correction factors for background and self-absorption were applied to the data.

Experiments to Verify Identity of Metabolites

This experiment was designed to collect sufficient amounts of metabolites of 2, 4-D from both susceptible plant (bean) and resistant plants (corn and bluegrass) for identification of these metabolites.

Approximately 200 plants of bean, corn, or bluegrass were germinated and grown in soil. Each plant was treated with approximately 100 microliters of 250 ppm non-radioactive 2, 4-D as the triethanol amine salt containing 0.1% surfactant Tween 20 in a foliar application when the plants attained a height of about eight inches. Plants were harvested two weeks after treatment.

The methods of isolation and purification of the metabolites were carried out in different ways. The 80% alcohol extraction was not used even though it provided a 99% recovery of radioactivity. Instead, extraction with 10% sodium carbonate was selected for three reasons. First, it extracted less interfering materials from plants. Secondly, eliminated the need to evaporate alcohol solvent.

Thirdly, prior experiments indicated that extraction with 10% sodium carbonate solution provided a good recovery of radioactivity. The procedure used was as follows:

Plants were extracted with hot, 10% sodium carbonate and heated on the steam bath with occasionally stirring for one hour. After filtration, sufficient hydrochloric acid was added to the extracts to raise the acid concentration to one molar acid. The extracts were then heated for three hours on a steam bath to achieve hydrolysis of the conjugated metabolites. At the end of hydrolysis, the extracts were filtered and extracted twice with ether. The ether extract was concentrated to a small volume under a stream of nitrogen gas on the steam bath. The water contained in the concentrated ether extract was removed by adding sodium sulfate. The water-free ether extract was filtered, and esterified with either diazomethane or diazopropane to give the respective methyl ether, methyl ester or propyl ether, propyl ester derivatives. The excess dizaoalkanes were removed by concentrating the ether under a stream of nitrogen.

In a preliminary purification, Woelm basic alumina, activity grade V, was used in column chromatography. An ether solution of the esterified metabolites was passed through a one by two inch column of the basic alumina. The column was washed with 300 ml of additional ether to elute the metabolites. An appreciable amount of

colored material was retained by the column.

The esterified metabolites were further purified by chromatography on thin-layer silica gel-G plates. Again, petroleum ether: ether:formic acid (50:50:2) was used as developer. The R_f zone of the compound was first determined using radioactive metabolites. The purified metabolites were obtained by scraping the silica gel from the area corresponding to the R_f zone of the radioactive compounds. The metabolites were eluted with distilled acetone in a soxhlet extractor by refluxing for 24 hours. The acetone extract was then concentrated to a small volume under vacuum. More diazomethane or diazopropane was added into the solution to ensure a complete esterification after refluxing.

Determination of the gas chromatographic retention times of the hydroxylated metabolites indicated that the methyl derivatives of 5-OH-2, 4-D and 4-OH-2, 5-D could not be separated. A number of column coatings were tested. However, neither polar nor non-polar coatings gave sufficient resolution to permit identification. (Glaze and Wilcox, 1966). This problem was overcome by the preparation of the propyl ether, propyl ester derivatives which can be separated by gas chromatography. These derivatives were prepared by treatment of the compounds with diazopropane.

In addition to the above identification, further confirmation of metabolite structure was obtained by determining the retention times

of mixed alkyl substituted compounds. These were prepared by first alkylating the phenolic and ester linkages with a propyl groups by treatment with diazopropane. After evaporation of the ether solvent, the residue was treated with 5 ml of 10% boron trifluoride in methanol. The mixture was heated in a sealed tube on the steam bath for three hours. The excess boron trifluoride was destroyed by the addition of 11 ml of water and the propyl ether, methyl ester was extracted with 2 ml of benzene. The benzene solution was then analyzed by gas chromatography.

RESULTS AND DISCUSSION

C^{14} Carboxyl Labeled 2, 4-D Experiments

The findings as to the extent of absorption of 2, 4-D by bean (susceptible plant) and corn (resistant plant) after different times of exposure are summarized in Table 1. Examination of the data in Table 1 reveals that an appreciable amount of 2, 4-D is readily absorbed by both types of plants from a foliar application. In three days following application of the 2, 4-D, corn plants absorbed approximately twice as much of the chemical as beans. However, in an extended exposure of seven days or longer, there were no significant differences between bean and corn plants in the amounts of 2, 4-D absorbed.

From prior studies, 2, 4-D was found to be poorly translocated by either type of plants. Approximately 1% to 2% of the absorbed radioactivity was translocated from tops to roots in foliar application, and from roots to tops in nutrient exposure. This was determined by separating the plants into tops and roots for separate extraction. Since the metabolism of 2, 4-D in the tops of the plants was of most interest in these studies, foliar application was used.

Ethanol was found to remove nearly all of the 2, 4-D and its metabolites from the plant tissues. Approximately 99% of the absorbed radioactivity was recovered in the 80% ethanol extraction.

Table 1. Extent of absorption of 2, 4-D by bean and corn plants after different length of exposure.

Length of exposure (days)	Bean plant		Corn plant	
	Total C^{14} absorbed (cpm)	% of absorption[1.]	Total C^{14} absorbed (cpm)	% of absorption[1.]
3 days	67,000	22	124,870	42
7 days	180,000	60	179,160	60
11 days	194,500	65	194,600	65

1. Percentage of absorption was obtained according to the following calculation:

$$\% \text{ of absorption} = \frac{\text{Total } C^{14} \text{ absorbed by plants (cpm)}}{\text{Total activities of 2, 4-D applied to the plants (cpm)}^{2.}} \times 100\%$$

2. Approximately 3×10^5 cpm of C^{14} carboxyl labeled 2, 4-D was applied to four plants.

In no case did the unextracted radioactivity exceed 3% of the total in the plant. Hence, there appeared to be no accumulation of an insoluble metabolites in the plant tissues.

Paper chromatography of the 80% alcohol extract indicated that the metabolism of 2, 4-D in corn plants was much more rapid than in bean plants. Using benzene:acetic acid:water (BAW) (2:2:1) as a developing solvent, two radioactive bands were found from bean plants, while only one radioactive band was observed from corn plants. By means of comparison of R_f values in paper chromatography, one of the bands from bean plant was further distinguished and found to coincide with the band of 2, 4-D. The second band from bean plant was similar to that of corn plant, having a very small R_f value. When butanol saturated with 1.5M ammonium hydroxide (BNW) was used as the developing solvent, the first radioactive band (zero R_f value in BAW developer) was found to have greater mobility. The band near the 2, 4-D band was quite broad, which suggested the presence of more than one compound. This band is probably comprised of various conjugates. A number of developing solvents were employed to effect resolution of the compounds in this band, but no definitive separation could be achieved.

The foregoing evidence indicated that the corn plant had conjugated all of the absorbed 2, 4-D with various constituents even as early as three days following application. On the other hand the bean

plant conjugated only approximately 60% of the absorbed chemical in the same time. In seven days, the bean plant had metabolized approximately 70% of the absorbed 2, 4-D through conjugation. At the end of 11 days, the bean plant had succeeded in conjugating approximately 88% of the absorbed herbicide. In the case of bean plant, complete metabolism of the 2, 4-D was never observed before death of the plant. Hence, it would appear that the resistance of plants to 2, 4-D may be associated with the rapidity of conjugation of the chemical. However, the actual mechanism of the action of 2, 4-D in killing plants is not known, although many different effects of 2, 4-D on plants have been observed. The extent of 2, 4-D metabolism by bean and corn plants is shown in Table 2.

As shown in Table 3 the conjugates of 2, 4-D and its metabolites migrate better with butanol saturated with 1.5M ammonium hydroxide (BNW) developing solvent in paper chromatography than with BAW developer. BNW developer was therefore selected to separate the un-metabolized 2, 4-D from its conjugates, and to partially remove the interferring material from these conjugated products.

To determine whether 2, 4-D was conjugated as sugar or peptide adducts, enzymatic hydrolysis as well as chemical hydrolysis of the 2, 4-D complexes was carried out in these studies. Thomas et al. (1964) demonstrated that 2, 4-D was converted principally into 1-0-(2, 4-dichlorophenoxyacetyl)-β-D-glucose (see Figure 2). Therefore

Table 2. Rate of 2, 4-D metabolism by corn, bean, and bluegrass plants.

Days following exposure		Percentage of radioactivity present as:				
		Free 2, 4-D	Conjugated products	Hydrolyzed conjugated product		
				2, 4-D	Major	Minor
Corn plant[1]						
3	crude extract[2]	0	100			
	Hydrolyzed			29	53	18
7	crude extract	0	100			
	Hydrolyzed			58	36	6
11	crude extract	0	100			
	Hydrolyzed			57	39	4
Bean plant						
3	crude extract	40	60			
	Hydrolyzed			37	48	15
7	crude extract	30	70			
	Hydrolyzed			57	37	6
11	crude extract	12	88			
	Hydrolyzed			64	33	3
Bluegrass plant						
7	crude extract	0	100			
	Hydrolyzed			55	39	6

1. No free 2, 4-D was observed in corn plant.
2. 80% alcohol extract.

Table 3. The R_f values of 2, 4-D and its conjugates in different developing solvents in paper chromatography.

Developing solvent	R_f values	
	2, 4-D	Conjugates
Benzene:acetic acid:water (2:2:1)	0.84	0
Butanol:1.5M ammonium hydroxide (saturated)	0.75	0.1

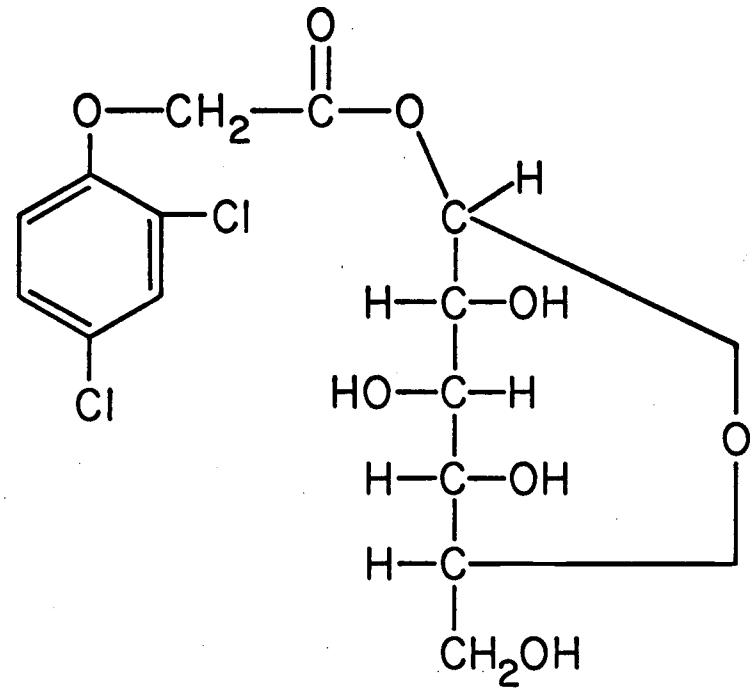


Figure 2. Structure of 1-O-(2,4-Dichlorophenoxyacetyl)-β-D-Glucose

β-glucosidase was chosen as one of the enzymes used in these studies. On the other hand, the formation of 2, 4-D conjugates as peptides has also been reported by a number of investigators (Klämbt, 1961; Bach and Fellig, 1961; Fang, 1958; Butts and Fang, 1956). A non-specific protease enzyme, pronase, was therefore used for the digestion since this would serve to distinguish between a peptide or sugar conjugate. Furthermore, from prior studies, it was found that either acid or alkaline hydrolysis resulted in loss of the broad band of conjugates and resulted in liberation of metabolites with the R_f values different from the 2, 4-D conjugates. Hence, either acid or base hydrolysis was carried out for the comparison of the extent of the hydrolytic reaction by enzymes.

Among a number of chromatographic methods and developing solvents, thin-layer chromatography with silica gel-G as support, and gas-liquid chromatography with a silicon column (either 7% OV-1 coating on 60/80 gas chrom Q or 20% SE-30 coating on gas chrom Q) achieved a definitive separation of the various possible hydrolytic products of 2, 4-D complexes. Chromatography of various possible hydroxy-analogs of 2, 4-D and 2, 4-D on thin-layer plates indicated that they were separable by this method of analysis. The R_f values are presented in Table 4. Gas chromatography of either methylated or propylated hydroxy-analogs of 2, 4-D and 2, 4-D also provided a good separation. Although 5-OH-2, 4-D and 4-OH-2, 5-D were not

Table 4. The R_f values of 2, 4-D, its hydroxy-analogs, its conjugates, and metabolites from extracts of bean, corn, and bluegrass plants in thin-layer chromatography.

Compounds	R_f Values
2, 4-D	0.85
6-OH-2, 4-D	0.88
5-OH-2, 4-D	0.66
3-OH-2, 4-D	0.70
4-OH-2, 5-D	0.75
4-OH-2, 3-D	0.53
Conjugates	0
Bean extracts	
Enzymic hydrolysis	0, .53, .74, .85
Chemical hydrolysis	.54, .75, .85
Corn extracts	
Enzymic hydrolysis	0, .54, .75, .85
Chemical hydrolysis	.54, .76, .85
Bluegrass extracts	
Enzymic hydrolysis	0, .53, .74, .85
Chemical hydrolysis	.53, .73, .85

separable in methylated treatment, they were separable in propylated treatment. By treatment of the samples with two diazoalkanes, all the hydroxyanalogs of 2, 4-D could be identified since the same two compounds were not inseparable with both treatments. Tables 5 and 6 present the retention times of 2, 4-D and its hydroxy-analogs.

Enzymatic digestion of the 2, 4-D complexes followed by ether extraction and comparison of the R_f values in thin-layer chromatography indicated that approximately 90% of the conjugates from bean, corn, and bluegrass plants may be hydrolyzed by the enzyme β-glucosidase. There was no significant amount of hydrolysis in the presence of pronase. This evidence would indicate that 2, 4-D and its metabolites were principally present in the plants as glucose adducts. This finding agrees with the work of Thomas et al. (1964a, b) quite well. Examination of the R_f values in thin-layer chromatography of 2, 4-D complexes digested with either acid or base demonstrated complete hydrolysis.

Thin-layer chromatography of the ether extracts showed the enzyme β-glucosidase yielded the same products as the chemical treatments. However, approximately 10% of the 2, 4-D or its derivative products were also found in the enzymic solution due to incomplete hydrolysis. Chromatography of the products of both enzyme and chemical hydrolysis indicated one major metabolite along with a considerable amount of 2, 4-D and one minor metabolite. The

Table 5. The retention times of methylated 2, 4-D, its hydroxy-analogs, and metabolites from extracts of bean, corn, and bluegrass plants in gas-liquid chromatography.[1]

Compounds	Retention time (minutes)
2, 4-D	2.3
6-OH-2, 4-D	4.5
5-OH-2, 4-D	5.7
3-OH-2, 4-D	5.1
4-OH-2, 5-D	5, 7
4-OH-2, 3-D	7.6
Bean extracts	2.3, 5.7, 7.6
Corn extracts	2.3, 5.7, 7.6
Bluegrass extracts	2.3, 5.7, 7.6

1. Temperature: 140° C

   Column: 7% OV-1 coating on gas chrom Q

   Detector: Microcoulometric (MC)

Table 6. The retention times of propylated 2, 4-D, its hydroxy-analogs, and metabolites from extracts of bean, corn, and bluegrass plants in gas-liquid chromatography.[1]

Compounds	Retention time (minutes)
2, 4-D	2.1
6-OH-2, 4-D	6.4
5-OH-2, 4-D	7.6
3-OH-2, 4-D	7.9
4-OH-2, 5-D	8.2
4-OH-2, 3-D	10.9
Bean extracts	2.1, 8.2, 10.9
Corn extracts	2.1, 8.2, 10.9
Bluegrass extracts	2.1, 8.2, 10.9

1. Temperature: 160° C

   Column: 7% OV-1 coating on gas chrom Q

   Detector: Microcoulometric (MC)

major metabolite was identical to that of 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2, 5-D) in all chromatographic tests. The minor metabolite proved to be identical with 2, 3-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2, 3-D). Both types of plants (bean and corn) yielded the same metabolites in the ratio of approximately 3:1, 6:1, and 10:1 respectively in 3, 7, and 11 days following treatment. Bluegrass plant also yielded the same metabolites in a ratio of approximately 6:1 in seven days following exposure. The amount of parent 2, 4-D in the conjugate varied from time to time, but was usually present as approximately 30% to 64% of the total radioactivity in the conjugates. As can be seen from Table 2, more 2, 4-D was conjugated in extended exposure. The percentage of radioactivity present as major and minor metabolites also varied with time and is also present in Table 2.

As noted earlier and shown by the data in Table 5 and 6, gas-liquid chromatography provided a good separation of 2, 4-D and its hydroxy-analogs when chromatographed as the methyl and propyl esters (Glaze and Wilcox, 1966). Using this technique it was possible to demonstrate the presence of the same metabolites in both types of plants following purification by thin-layer chromatography. Again, as in the case of thin-layer chromatography, one major metabolite along with a large amount of 2, 4-D and one minor metabolite were observed on the chromatogram. The retention time of each of the

major and minor products were identical with either the methyl ester or propyl ester of the authentic 4-OH-2, 5-D and 4-OH-2, 3-D respectively. Determination of the areas under the chromatographic peaks of these two metabolites, again indicated that they were present in a ratio of approximately 3:1, 6:1, and 10:1 respectively in 3, 7, and 11 days following exposure.

As can be seen in Table 5, the retentions of the methyl esters of 5-OH-2, 4-D and 4-OH-2, 5-D are identical, and that the retention time of the major metabolite is similar. Hence, it was not possible to identify this major metabolite in the methyl ester form. Fortunately, the thin-layer chromatography as well as the gas chromatography of the propyl ester provided a technique of separation of 4-OH-2, 5-D from 5-OH-2, 4-D. Thus it was possible to establish that the major metabolite is 4-OH-2, 5-D. However, in order to further validate the conclusion that the major metabolite is 4-OH-2, 5-D, gas chromatography of propyl ether, methyl ester of the metabolites (Glaze and Wilcox, 1966) was carried out.

Thus far, the metabolic pathways of 2, 4-D in either susceptible or resistant plants seem to through conjugation of 2, 4-D as glucose adducts, followed by or simultaneous replacement of chlorine at the 4-position of the aromatic ring by a hydroxy group, accompanied by a shift of the chlorine to an adjacent position on the aromatic ring. The chlorine shift was predominantly to the 5-position of the aromatic

ring. This position may be favored because of less steric hinderance.

If the conjugation with glucose of the parent 2, 4-D occurred primarily through the carboxyl group, the hydroxymetabolites should be attached to the glucose through the carboxyl group. However, following hydroxylation of the ring of 2, 4-D there is now a second group that may react to form a conjugate. Hence, it is very possible to have the metabolites bound to the glucose through the phenolic group of the 2, 4-D as well.

It has been demonstrated by Broadhurst et al. (1966) working with 2-methoxy-3, 6-dichlorobenzoic acid that following hydroxylation, conjugation occurred through the phenolic group. These findings were based on the following experiment. The conjugated metabolite was treated with diazomethane prior to hydrolysis in order to determine whether the phenolic group was free or bound in the conjugated metabolite. If the phenolic group were free, it would be methylated during the treatment. On the other hand, if the phenolic group were bound in the conjugated metabolite, then the R_f value of the metabolite following hydrolysis of the conjugated metabolite should be the same as if no prior diazomethane treatment had been used. The R_f value of the metabolite was found to be the same as if no diazomethane treatment had been used. Hence, it was concluded that the phenolic group was conjugated and unavailable for methylation in the parent metabolite. In order to confirm these findings, a

free hydroxylated product was methylated, and then saponified by adding sodium hydroxide. Following extraction, the metabolite was chromatographed. The R_f value was no longer identical to that of the original hydroxylated product. This indicated that the hydroxyl substituent of the unconjugated material had been methylated, which is in contrast to that found with the conjugated material.

In repeating this experiment on the 2, 4-D conjugates similar results were obtained. Therefore, it appears that the phenolic group as well as the carboxyl group of the 2, 4-D metabolites are involved in the glucoside conjugation. On the basis of this and foregoing data a scheme of metabolic pathways of 2, 4-D in plants is proposed in Figure 3.

Another path in the metabolism of 2, 4-D in plants is that which results in decarboxylation. It was necessary therefore to investigate the decarboxylation of the chemical in order to assess the importance of this path. Decarboxylation of 2, 4-D by plant tissues has been reported in several studies (Holley, 1951; Weintraub et al., 1950, 1952, 1954, 1956; Bach and Fellig, 1959, 1961; Edgerton, 1961; Edgerton and Hoffman, 1961; Luckwill and Lloyd-Jones, 1960 a, b). The extent of decarboxylation varied from species to species; and different positions of C^{14} labeled 2, 4-D also resulted in variation in the extent of decarboxylation. If this reaction were to occur where the C^{14} label is in the carboxyl group, the metabolites formed

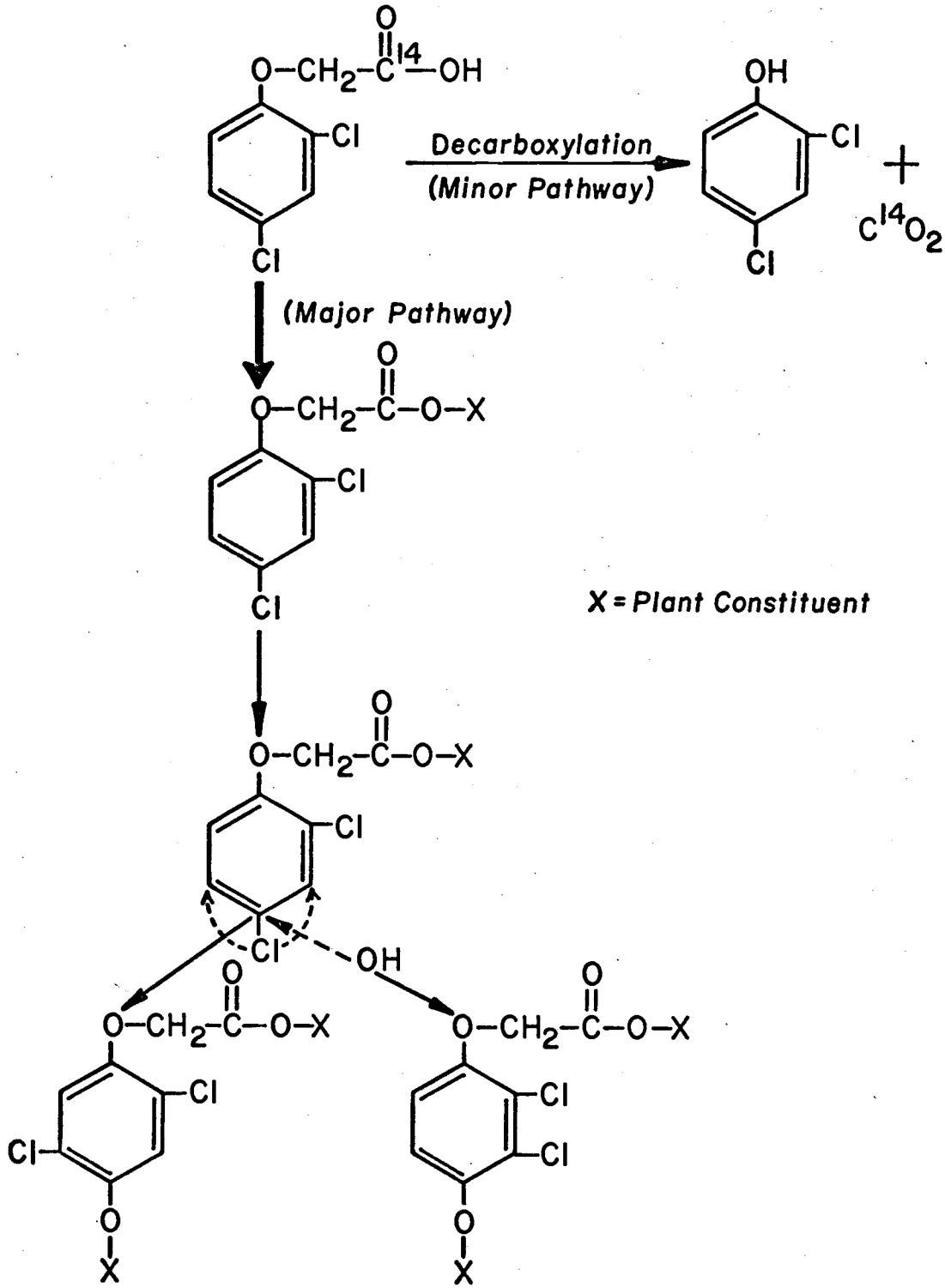


Figure 3. A Summary of Proposed Metabolic Pathway of 2,4-D in Plants.

other than $C^{14}O_2$ could not be detected by means of radioactivity.

Accordingly, the $C^{14}O_2$ was collected from the carboxyl labeled 2, 4-D (Weintraub et al., 1950, 1952) treated plants to compare the extent of decarboxylation from both susceptible and resistant plants. Approximately 3.2% of the absorbed radioactivity was obtained as $C^{14}O_2$ from bean plants in two days while only 1.5% of the total C^{14} carboxyl labeled 2, 4-D was evolved as $C^{14}O_2$ from corn plants. Although bean plants decarboxylated approximately two times as much $C^{14}O_2$ as corn plants, there was no significant difference in decarboxylation of 2, 4-D between these two species of plants. Obviously, the amount of 2, 4-D metabolized via the decarboxylation process is not particularly significant. The resistance of the plants to 2, 4-D does not therefore appear to be associated with the rapidity or extent of decarboxylation of the herbicide. Rather the major route of metabolism of 2, 4-D by plants is conjugation followed by hydroxylation accompanied by a shift of chlorine with decarboxylation being a minor path of degradation. Table 7 presents the extent of decarboxylation by both types of plants.

Experiments to Verify Identity of Metabolites

Further demonstration that the major metabolite is identical in bean, corn, and bluegrass plants, that is 4-OH-2, 5-D was sought using non-labeled 2, 4-D for treatment.

Table 7. The release of C^{14} as $C^{14}O_2$ in two days by bean and corn plants treated with C^{14} carboxyl labeled 2, 4-D.

	Bean plants (cpm)	Corn plants (cpm)
Total C^{14} in $BaCO_3$ ($C^{14}O_2$ precipitated as $BaCO_3$)	2.78×10^4	2.38×10^4
Total C^{14} in 80% alcohol extract[1].	8.35×10^5	1.59×10^6
Percent of the absorbed radioactivity liberated as $C^{14}O_2$[2].	3.2	1.5

1. Total C^{14} in plant tissues was extracted by 80% ethanol.

2. Percent of the radioactivity liberated as $C^{14}O_2$ was obtained according to the following calculation:

$$\% \text{ of the absorbed activity liberated as } C^{14}O_2 = \frac{\text{Total } C^{14} \text{ in } BaCO_3}{\text{Total } C^{14}O_2 \text{ in } BaCO_3 + \text{Total } C^{14} \text{ in alcohol extract}}$$

Accordingly, an appropriate amount of the major metabolite of 2, 4-D from both susceptible (bean), and resistant (corn and bluegrass) plants were collected. This was accomplished by extraction of the metabolite from plants treated with unlabeled 2, 4-D with 10% sodium carbonate solution. As indicated earlier, 10% sodium carbonate extraction provided a good recovery of the herbicide and metabolites from plant tissues, and picked up less interfering materials. Following hydrolysis, ether extraction and derivatization, the metabolite was subjected to purification by alumina column containing 15% water, followed by thin-layer chromatography before final identification of the metabolite by gas chromatography.

From the prior studies, it was found that 2, 4-D and its metabolites degraded in the acidic ether extract. However, the ester form of these chemicals was quite stable. The metabolite was therefore derivatized right after ether extraction to avoid any degradation of the compound.

Since the derivatized extracts contained a considerable amount of plant constituents, further purification was required. The metabolites were largely separated from interfering substance by column chromatography using basic alumina containing 15% water. Part of colored material was absorbed on the column as a sodium salt, while most of the esterified metabolites (approximately 85%) passed the column.

It became evident that a further clean-up step was required, since some of the interfering material also passed through the column along with the metabolites. Thin-layer chromatography of the extract from plant containing C^{14} methylated metabolites indicated that the methylated metabolites migrated near the solvent front (0.95 R_f), while most of the interferring material remained on the original spot. The second step of purification employed was thin-layer chromatography. This clean-up step provided 95% recovery of the metabolites.

After the metabolites had been purified, gas chromatography was used for further confirmation of the identity of the metabolites. This was done by determination of the retention times of the propyl ether, methyl esters of the metabolites and comparing them to those of authentic compounds. These data are presented in Table 8. It will be noted that 4-OH-2, 5-D, 4-OH-2, 3-D, and 2, 4-D are the metabolites found.

The retention times of the methylated and propylated metabolites from extracts of both types of plants treated with non-labeled 2, 4-D agreed very well with that of the metabolites from C^{14} carboxyl labeled 2, 4-D treated plants. Each of methylated, propylated, and methylated following propylated metabolites of major and minor were present in a ratio of approximately 10:1 in all types of plants. This finding was identical with the work of Thomas et al. (1964b).

Table 8. The retention times of the methyl ester, propyl ether of 2, 4-D, its hydroxy-analogs, and metabolites from extracts of bean, corn, and bluegrass plants in gas-liquid chromatography. [1]

Compounds	Retention time (minutes)
2, 4-D	1.7
6-OH-2, 4-D	5.6
5-OH-2, 4-D	7.0
3-OH-2, 4-D	6.9
4-OH-2, 5-D	7.4
3-OH-2, 3-D	10.0
Bean extracts	1.7, 7.4, 10.0
Corn extracts	1.7, 7.4, 10.0
Bluegrass extracts	1.7, 7.4, 10.0

1. Temperature: 150° C

   Column: 7% OV-1 coating on gas chrom Q

   Detector: Microcoulometric (MC)

Thus, by means of retention times in gas chromatography, the major metabolite was further identified as 4-OH-2, 5-D. The minor metabolite again appeared as 4-OH-2, 3-D. They were present in a ratio of approximately 10:1 in two weeks following application of 2, 4-D.

SUMMARY AND CONCLUSIONS

2, 4-D is one of the most active of the selective herbicides and has been found to kill numerous broad-leaved plants. Although this chemical has received more extensive study than any other herbicide, its complete metabolic fate is still not fully elucidated. Further, there has been no agreement on extent, rate, and path of degradation of this chemical, and no systematic comparison of the metabolism of 2, 4-D in resistant and susceptible plants has been made. Therefore, the resistance of plant to 2, 4-D was left in doubt. This study was carried out to compare the rates and paths of metabolism of 2, 4-D in resistant and susceptible plants.

Both susceptible (bean) and resistant (corn and bluegrass) plants were treated with C^{14} carboxyl labeled 2, 4-D from a foliar application. Plants were harvested after 3, 7, and 11 days following application of the chemical. Approximately 99% of the radioactivity was removed by ethanol, and no significant amounts of unextracted chemical were found to be accumulated in plants. Free 2, 4-D which was not metabolized by plants was removed from the extract and the 2, 4-D and its metabolites conjugates were partially purified by means of paper chromatography. After hydrolysis of the conjugates by either enzyme, acid, or base, the metabolites were analyzed by thin-layer chromatography. The metabolites were separated,

methylated, and propylated following purification by thin-layer chromatography, and the retention times were determined by gas-liquid chromatography.

Further confirmation for the identity of metabolites was carried out by using cold 2, 4-D treated plants. Similarly, the metabolites were isolated from the extract of plant following hydrolysis, purified by column followed by thin-layer chromatography, and identified by each of retention times of methyl ester, propyl ester, and propyl ether, methyl ester.

It was found that 2, 4-D was readily absorbed by both types of plants. Approximately 22% and 42% of the applied 2, 4-D was absorbed by bean and corn plants respectively in three days following application of the chemical. In an extended exposure of seven days or longer, there was no significant difference between bean and corn plants in the amount of 2, 4-D absorbed. Approximately 60% and 65% of 2, 4-D was absorbed by both types of plants in 7 and 11 days respectively.

Paper chromatography revealed that 2, 4-D was metabolized in corn plant much more rapidly than bean plant. In three days following foliar application of 2, 4-D, corn plant had metabolized all of the absorbed chemical, and no free 2, 4-D was found. On the other hand, approximately 40% of the 2, 4-D remained as free chemical in bean plants in the same time. The bean plants had metabolized approximately 70% of the absorbed 2, 4-D in seven days, and approximately

88% at the end of 11 days. No complete metabolism of the 2, 4-D was observed before the death of bean plants. Hence, the resistance of plants to 2, 4-D may be associated to the rapidity of conjugation of the chemical.

Two metabolites beside the parent 2, 4-D were found as glucose adducts in both types of plants in a ratio of approximately 10:1 in two weeks following application of the chemical. By means of R_f value in thin-layer chromatography and retention times in gas-liquid chromatography the major and minor metabolites were identified as 4-OH-2, 5-D and 4-OH-2, 3-D respectively. A significant portion of the absorbed 2, 4-D appeared as one of the conjugated products.

Relatively low rates of $C^{14}O_2$ evolution by plants treated with carboxyl-labeled 2, 4-D was observed in the case of corn plant as well as bean plant. The corn plant decarboxylated 2, 4-D to the extent of 1.5% of the absorbed herbicide in two days while bean plant decarboxylated 3.2% at the same time period. However, there were no statistically significant differences in decarboxylation of 2, 4-D between these two species of plants. The resistance of the plants to 2, 4-D does not, therefore, appear to be associated with the extent of decarboxylation of the herbicide.

Apparently the major route of metabolism of 2, 4-D by plants is conjugation followed by hydroxylation accompanied by a shift of

chlorine with decarboxylation being a minor path of degradation. The resistance of plant to 2, 4-D thus appears to depend upon the rapidity of conjugation of the absorbed chemical as glucose adducts. No significant difference in the rate of absorption was observed in the different types of plants. Therefore, the detoxification mechanism is not very likely to be associated with the rate of absorption, but rather depends upon the rate of metabolism. The incomplete metabolism of 2, 4-D and the persistence of free 2, 4-D in susceptible plants may be a major factor in the lethal action of this compound.

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