Metabolites of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Bean Plants

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Microcoulometric gas chromatography was employed to investigate the metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in bean plants. The controversial ether-soluble substance in the plant extracts was proved conclusively to be the herbicide itself. The ether-insoluble fraction yielded 2,4-D and another chlorinated compound upon acid hydrolysis, but no 2,4-dichlorophenol or its derivatives could be detected.

ALTHOUGH 2,4-dichlorophenoxyacetic acid (2,4-D) remains the herbicide most widely used in the United States, little more is known now about its metabolites and the mechanism by which it stimulates plant growth than a decade ago. This is particularly true with respect to the chemical identity of the 2,4-D relatives which have been detected for many years in herbicide-treated plants.

During the past 13 years, the metabolism of this compound has received the attention of a number of investigators (*2, 7–10, 13*). Although some eight species have been examined, the bean plant has provided most of the existing information. However, no chlorine-containing compound previously has been positively identified in extracts of treated plants. To date, only two methods have been employed for the detection and identification of 2,4-D metabolites in higher plants. Bioassay has provided a small amount of information, but the isotopic tracer technique has led to almost all of our present knowledge.

However, the use of tracers has been restricted to study of the side-chain; only 2,4-D-carboxyl-C^{14} and 2,4-D-methylene-C^{14} have been employed. It would be of considerable interest to be able to determine the fate of the aromatic ring and its substituents, in addition. Furthermore, although the sensitivity with which radioactive isotopes may be detected has been of great value, some means must be provided for the actual isolation and purification of sufficient quantities of 2,4-D metabolites to permit conclusive chemical identification.

The present investigation essayed to accomplish these aims by searching for chlorine-containing organic compounds in 2,4-D-treated plants. Gas chromatography was used for resolution of the plant constituents, and those containing chlorine were detected specifically by combustion and microcoulometry.

Experimental

Materials and Equipment. 2,4-D was purified by successive recrystallization from benzene, ethanol, and water until a sharp m.p. of 139° C. (corr.) and homogeneity toward gas chromatography were attained. Authentic 2,4-D methyl ester was prepared from the pure acid by reaction with diazomethane in ether; 2,4-dichloroanisole and 2,4-dichlorophenol were purchased from Matheson Coleman & Bell; and 5-hydroxy-2,4-dichlorophenoxyacetic acid was generously provided by Wayne Thornburg.

Chlorinated compounds were resolved by gas chromatography of an aliquot of a benzene or ether solution of the sample on a 6-foot column containing 20% Dow 11 silicone oil on Chromosorb P at the appropriate temperature indicated in the text. Temperature ranges indicate difficulty in close oven regulation. Chlorine content was measured with a microcoulometric detector (Dohrmann

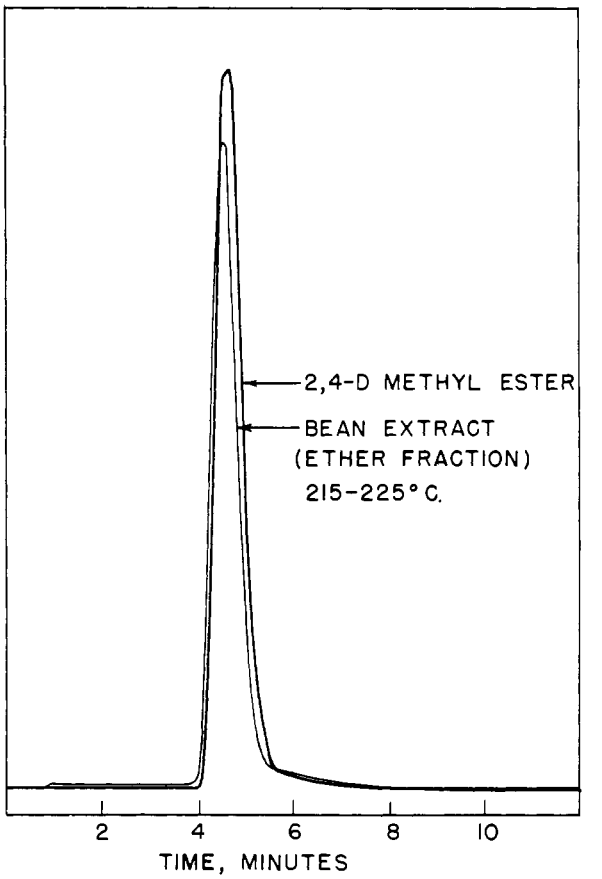

Figure 1. Gas chromatogram of methylated "Extract A"
Microcoulometric detector

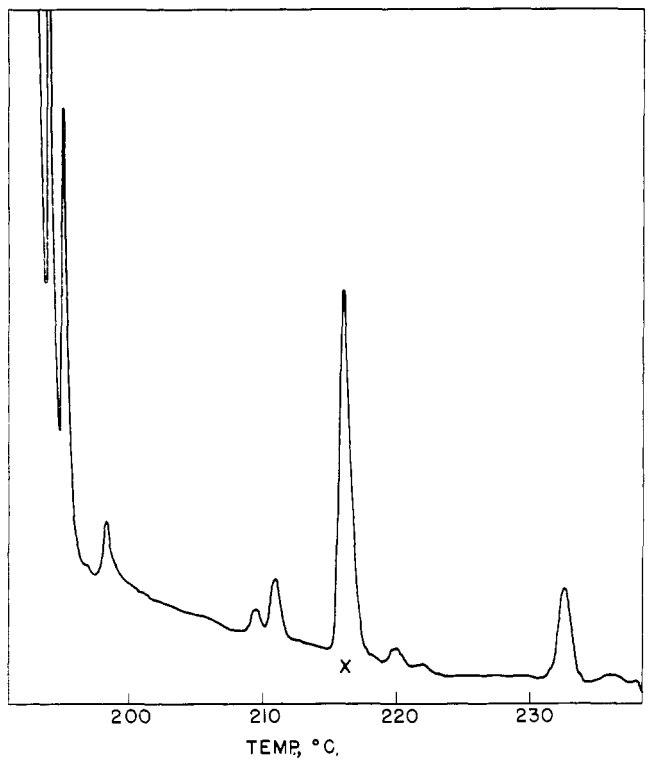

Figure 2. Gas chromatogram of methylated "Extract A"

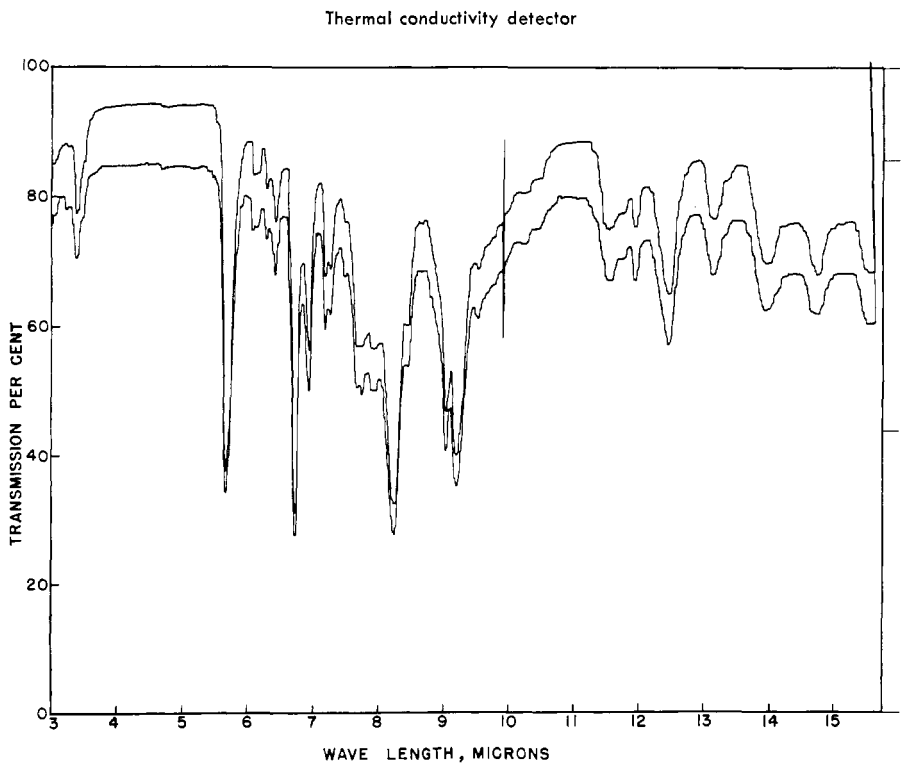

Figure 3. Infrared spectra of 2,4-D methyl ester (upper line) and peak X of methylated "Extract A" (lower line)

Instruments Co., Inc., Palo Alto, Calif.) (*5*). Blank runs on solvents and on extracts from untreated control plants were carried out in all applicable cases.

Preparative gas chromatography was carried out with an F and M Model 500 programmed temperature gas chromatograph equipped with a thermal conductivity detector. A 2-foot column containing 2.5% SE-30 silicone oil on Chromosorb P was employed; fractions were collected on ethyl acetate-soaked glass wool plugs held in 6-mm. glass tubes; the plugs subsequently were extracted with warm ethyl acetate; and solvent was removed by evaporation. Infrared spectra were measured in thin films with a Perkin-Elmer Model 221 Spectrophotometer, and ultraviolet spectra were obtained with a Beckman Model DK-2A recording spectrophotometer.

Extraction. Bean plants (*Phaseolus vulgarus* L., var. Red Kidney) were germinated and grown in wet vermiculite without nutrients under standard greenhouse conditions. On about the twelfth day after planting, when the primary leaves were fully expanded, each leaf was treated with approximately 200 μg. of the carefully purified 2,4-D as a 0.1% solution in 95% ethanol containing 1% of Tween 20 as a wetting agent. Up to 800 plants were employed in each experiment.

On the 4th day after treatment, the plants were cut at ground level, leaves and most of each petiole were removed, and the stems were wiped to remove any external trace of herbicide. They were then homogenized with 80% ethanol in a Waring Blendor, the mixture was filtered by suction through a pad of Celite, and the clear filtrate was evaporated under reduced pressure at room temperature.

The residue was dissolved in a moderate volume of water and continuously extracted with redistilled ethyl ether for at least 24 hours until subsequent examination by gas chromatography indicated that no extractable chlorine compounds remained. The ether extract could be termed "Extract A," and the remaining aqueous layer "Residue A."

Results and Discussion

When "Extract A" was analyzed on the Dohrmann instrument, no chlorine-containing substances could be detected up to the temperature maximum of the instrument (285° C.) within a period of 30 minutes. (For comparison, 2,4-D methyl ester had a retention time of 3 minutes at 265° C.) After removal of the ether from "Extract A," the residue was dissolved in redistilled benzene and esterified by treatment with excess diazomethane in ether (*4*). This time, analysis revealed a single strong peak with a retention time identical to that of the 2,4-D methyl ester standard (Figure 1).

The methylated mixture was resolved on the F and M gas chromatograph (Figure 2). Only peak *X*, corresponding in retention time to 2,4-D methyl ester, could be demonstrated to contain chlorine by microcoulometry. This peak was collected cleanly in four separate runs; the collections were pooled, rechromatographed, and recollected. A total of approximately 800 μg. of pure compound was obtained.

Comparison of the infrared spectrum of this substance with that of authentic 2,4-D methyl ester established its identity (Figure 3). The identical ultraviolet spectra of the two samples provided further proof.

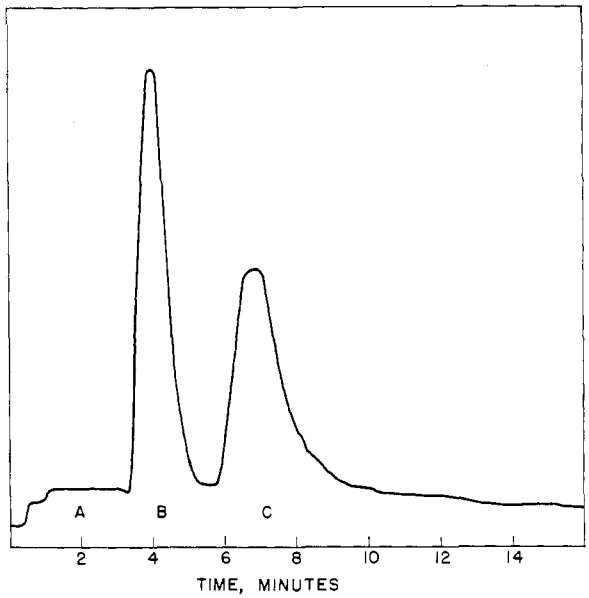

Figure 4. Microcoulometric gas chromatogram of methylated ether extract of hydrolyzed "Residue A" (260°–232° C.)

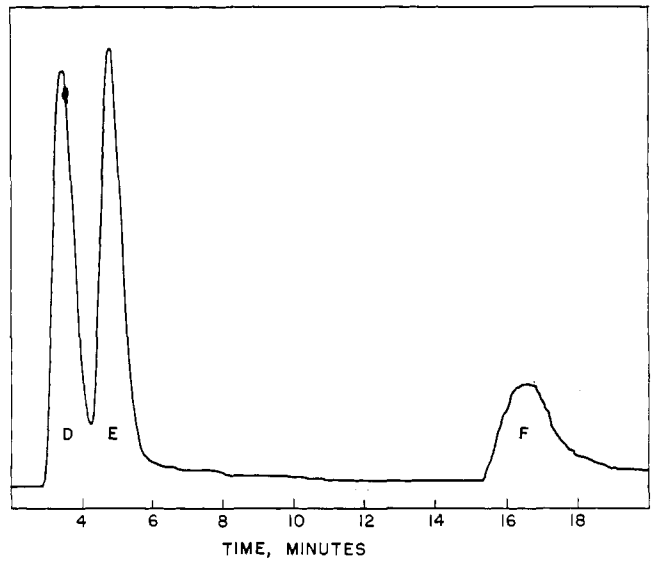

Figure 5. Microcoulometric gas chromatogram of 2,4-dichlorophenol (*D*), 2,4-dichloroanisole (*E*), and 2,4-D methyl ester (*F*), 2 μg. each at 202°–175° C.

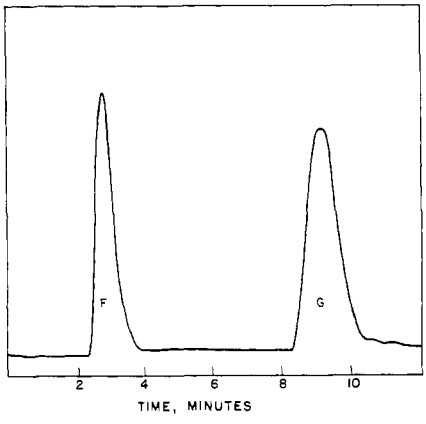

Figure 6. Microcoulometric gas chromatogram of 2 μg. of 2,4-D methyl ester (*F*) and 5 μg. of 5-methoxy-2,4-D (*G*) at 255°–265° C.

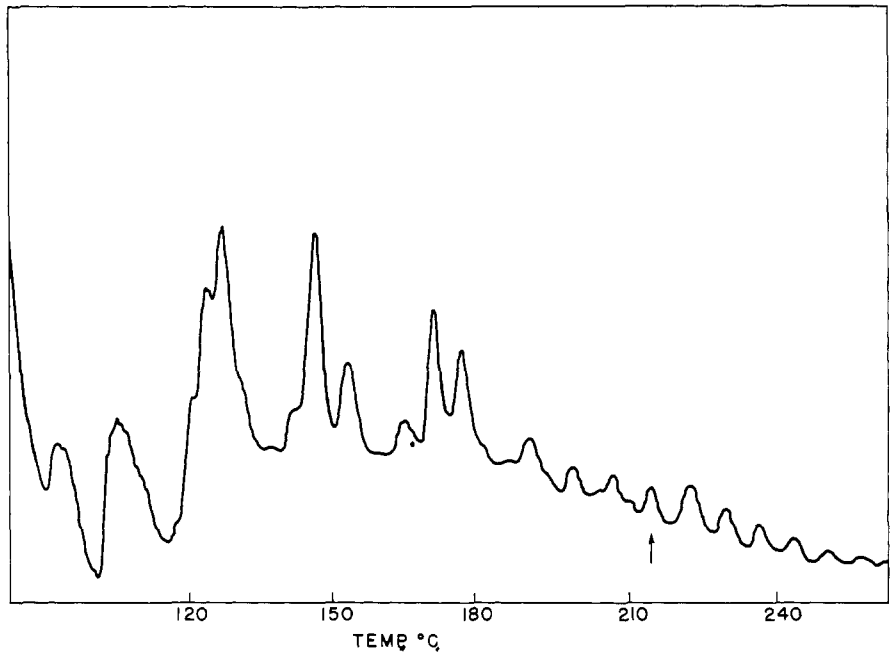

Figure 7. Gas chromatogram of the methylated ether extract of hydrolyzed "Residue A" (thermal conductivity detector)

Arrow indicates approximate location of 2,4-D methyl ester

Contrary to the suggestion of Bach (*2*), free 2,4-D appears to be the major chlorine-containing ether-soluble constituent of the bean plant 4 days after treatment, and certainly it is the only one to form a volatile derivative. Neither 2,4-dichlorophenol nor 2,4-dichloroanisole could be detected, although the microcoulometric method was extremely sensitive toward them (Figure 5) and fortification experiments revealed that they were not lost during the extraction and subsequent manipulations.

The thoroughly extracted aqueous fraction ("Residue A") was shown to be free of any volatile chlorine compounds even after methylation. This fraction was hydrolyzed by boiling with dilute sulfuric acid, and the acidic solution was continuously extracted with ether, dried, and evaporated. Again, no volatile chlorinated substances could be detected.

However, after methylation with diazomethane, at least three such compounds were found (Figure 4). The first poorly resolved peak (*A*) corresponds roughly to that of the 2,4-dichloroanisole, which would be formed from any phenol present. The amount would have to be extremely small considering the sensitivity of response to this substance (Figure 5). Luckwill and Lloyd-Jones (*12*) detected a significant increase in the steam-volatile phenols from strawberries treated with 2,4-D; they attributed this to the formation of 2,4-dichlorophenol, although only the nonspecific Folin-Dennis reagent was used for detection.

The second peak (*B*) represents 2,4-D methyl ester. Thus, a considerable portion of the 2,4-D absorbed by the plant is apparently converted without any basic structural change into a

simple water-soluble, ether-insoluble derivative that is readily hydrolyzed. Although amino acids have been implicated in the formation of such compounds, as in the case of 2,4-dichlorophenoxyacetylaspartic acid in pea stem sections (*1*), the amides so formed generally are soluble in ether. More likely, this is a sugar ester such as the glucose ester suggested recently by Klämbt (*11*).

The third chromatographic peak (*C*) likewise represents the chlorine-containing part of a major metabolite of 2,4-D, although in this case a basic structural change apparently has been involved. The metabolite again most probably exists in vivo as a sugar derivative. Extraction experiments show that its ether-soluble hydrolysis product is an acid, but it could contain more than one diazomethane-reactive group. Its methyl derivative is somewhat less volatile than 2,4-D methyl ester, but more volatile than methylated 5-hydroxy-2,4-D (Figure 6). Of course, it might indeed be one of the other two ring-hydroxylated 2,4-D's, although Bach (*2*) could not detect 6-hydroxy-2,4-D during his experiments. The isolation and identification of this metabolite are in progress.

The results of the present investigation are consistent with the common pattern which emerges upon close examination of the previous literature. The major ether-soluble constituent of 2,4-D-treated bean plants is indeed the parent herbicide itself. Two major water-soluble, ether-insoluble derivatives are present, one of which may be hydrolyzed with acid to 2,4-D. Minute amounts of 2,4-dichlorophenol may possibly be present, but probably many of the previously reported "metabolites" are artifacts.

The present application of microcoulometric gas chromatography appears to offer major advantages for study of the metabolites of many types of chlorine- and sulfur-containing compounds. A comparison of the well-defined results obtained through microcoulometry (Figure 4) with the picture obtained from an identical sample when thermal conductivity detection was employed (Figure 7) is indicative of the utility of the method. In addition to sensitivity, specificity, and quantitative accuracy (*5*), this technique provides information on experimental conditions which may be readily applied to the isolation of the detected metabolites, as illustrated above.

A number of important aspects of 2,4-D metabolism currently are under investigation in this laboratory by use of the present microcoulometric procedure. Of particular interest is the identification of the water-soluble "Unknown 3" of Fang and Butts (*6*). This substance constitutes the principal 2,4-D metabolite in corn, wheat, pea, and tomato plants. The use of the waste leaves and stems as animal fodder makes the identity of "Unknown 3" important from a toxicological viewpoint. The possible presence of this substance in treated pasture grass and hay likewise lends unusual importance to its identification.

Knowledge of the chemical nature of these metabolites obviously is important from the viewpoint of pesticide residue analysis. If any significant part of the 2,4-D applied to a plant were converted to a stable but readily hydrolyzed derivative, or if such metabolites were found to be interconvertible in vivo with the parent herbicide, the results of trace analysis of 2,4-D residues may be seriously affected.

The resistance or susceptibility of a particular plant or variety to hormone-type herbicides may be better explained when the exact means of detoxication are understood. The relative rates of formation of metabolic detoxication products as well as the sequence of their formation might be expected to have significance in this regard.

Finally, knowledge of the chemical nature of 2,4-D metabolites may shed light on the mechanism by which plant growth substances exert their effects. Although there now exists a considerable body of knowledge about the physiological processes affected by synthetic hormones, the mechanism by which cell elongation is stimulated remains obscure. The water-soluble fraction from 2,4-D-treated beans was previously found to be inactive in the oat first-internode bioassay for growth promoters (*3*). The possible conversion of an inactive derivative into the highly active 2,4-D by simple hydrolysis may provide a structural key to both mode of action and synthesis of more specific herbicides.

Acknowledgment

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