Enzymatic Cleavage of the Ether Bond of 2,4-Dichlorophenoxyacetate

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A soluble enzyme preparation obtained from a soil *Arthrobacter* sp. catalyzes the cleavage of the ether linkage of 2,4-dichlorophenoxyacetate (2,4-D). The cleavage appears to proceed by an oxidative mechanism to yield 2,4-dichlorophenol. The enzyme preparation converts acetate-labeled 2,4-D to alanine and a volatile product. Acetate and glycolate are not metabolized, but glyoxylate is metabolized at a much faster rate than 2,4-D. It is proposed that glyoxylate is the initial product formed enzymatically upon cleavage of the side chain from the 2,4-D molecule, and that alanine is produced following the condensation of two molecules of either glyoxylate or glycine.

The phenoxyacetates have been widely used as selective herbicides, and a knowledge of the mechanism of their detoxication is therefore important to an understanding of the fate of the chemicals in nature. The first enzymatic studies of the cleavage of the ether linkage of phenoxy herbicides were reported by Loos *et al.* (1967a), who identified 2,4-dichlorophenol as the initial aromatic product formed from 2,4-dichlorophenoxyacetate (2,4-D) by a soil bacterium. Helling *et al.* (1968) observed that resting cells of the same organism converted ^{18}O ether-labeled phenoxyacetate to phenol, the latter containing all of the original ^{18}O label.

The product formed from the acetate moiety of 2,4-D has never been identified, however. The acetate side chain is apparently not decarboxylated, inasmuch as enzyme preparations converting 2,4-D to 2,4-dichlorophenol were inactive on 2,4-dichloroanisole (Loos *et al.*, 1967a). MacRae and Alexander (1963) reported that a *Flavobacterium* sp. grown on 4-(2,4-dichlorophenoxy)butyrate liberated the free fatty acids from 2,4-dichlorophenoxyalkanoates having alkanoic acid moieties with three or more carbon atoms, the data suggesting either a reductive cleavage or a hydrolytic cleavage followed by a reduction. The *Flavobacterium* sp., however, was not active on 2,4-D.

An oxidation of the methylene-carbon of the 2,4-D side chain followed by a cleavage of the aliphatic moiety might also occur. Gaunt and Evans (1961) isolated oxalate from a pseudomonad culture grown in a medium containing 4-chloro-2-methylphenoxyacetate, but they did not determine the source of the oxalate. Glyoxylate might be formed from the side-chain of 2,4-D, for example, by reactions similar to those studied by Axelrod (1956) and Cartwright and Smith (1967).

In the present study, information is presented on the cleavage of the ether linkage of 2,4-D by soluble enzymes derived from an *Arthrobacter* sp.

MATERIALS AND METHODS

The phenoxyacetate-degrading *Arthrobacter* sp. was grown, harvested, and the cells disrupted by the procedures of Loos *et al.* (1967a). Whole cells and larger components of broken cells were removed by centrifuging the suspension at 20,000 × G for 30 minutes to yield a crude cell extract. The remaining particulate components were removed by centrifuging the supernatant at 140,000 × G for 90 minutes to yield a soluble fraction. This soluble enzyme preparation, which was stored at −10° C. for no more than 2 days, was used in all experiments. Because the preparation catalyzing the initial 2,4-D detoxication was generally labile, extracts were always kept cold and were incubated in solutions containing approximately $10^{-3}M$ cysteine.

The reaction mixtures were incubated for 3 hours at 28° C. with shaking, unless otherwise stated. The buffer used was 0.02*M* potassium phosphate, pH 7.3. All substrates were dissolved in phosphate buffer and neutralized with NaOH prior to incubation.

A dual-channel Packard Tri-Carb scintillation spectrometer, Model 3002, was employed in isotope studies, the counter being operated at gain 8.7 with window settings of 0.5 to 10.0. Thin-layer chromatograms were sectioned and placed in scintillation vials containing 10 ml. of Spectrafluor (Nuclear-Chicago, Des Plaines, Ill.) diluted with toluene. Aqueous samples were counted in 15 ml. of Bray No. 2 solution (Bray, 1960), but with the substitution of dimethyl-POPOP for POPOP. Counts of incubation mixtures initially containing labeled 2,4-D were made in the presence of protein by suspending the protein-containing aliquot in a Bray No. 2 solution with 4% thixotropic gel powder.

The radioactive product generated from the aliphatic moiety of 2,4-D was identified by cochromatography on both Eastman Chromagram silica gel sheets (Eastman Organic Chemicals, Rochester, N. Y.) without indicator and Brinkmann (Westbury, N. Y.) MN-Polygram cellulose sheets. The sheets were developed in an Eastman Chromagram chamber. The standard amino acids were detected with a 0.2% ninhydrin-ethanol spray.

The area containing unknown radioactive products was cut into approximately 1.5-sq. cm. sections, and the sections were placed in a scintillation vial for assay.

Glyoxylate was determined by the method of Trijbels and Vogels (1966), and glycolate was measured by the procedure of Lyle and Sani (1965). The latter procedure was not always reliable at high protein concentrations because of background color. The possible formation of glycolaldehyde was assessed by the method of Coe (1965). Chloride release was assayed quantitatively by the method of Bergmann and Sanik (1957), and phenol production was measured by the 4-aminoantipyrine procedure (Loos *et al.*, 1967b). Protein concentration was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

Sodium glyoxylate-1-^{14}C (8.44 mc. per mmole) and 2,4-D labeled in the 1- and 2-position of the side chain (12.1 and 21.7 mc. per mmole, respectively) were obtained from Nuclear-Chicago. Glycolic acid-2-^{14}C (34.0 mc. per mmole) was

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Table I. Enzymatic Conversion of Carboxyl- and Methylene-Carbons of 2,4-D to Volatile Products and Release of Chloride

Preparation[a]	Volume of Preparation, Ml.	Incubation Time, Hours	Methylene-Carbon Lost, %	Carboxyl-Carbon Lost, %	Chloride Formed, %
A	0.5	3	0	30	60
B	0.8	4	0	28	51
C	0.8	3	0	76	100
D	0.4	2	0	15	25

[a] Incubation mixtures contained 1.0 μmole of substrate in 0.2 ml. of solution plus the extract. Preparations A, B, and D had been stored at −10° C. prior to use, while C was used immediately after preparation.

provided by Volk Isotopes (Burbank, Calif.). No radiochemical impurities were detected by thin-layer or paper chromatography of these chemicals. Radioactive sodium acetate (99% radiopurity) was obtained from Calbiochem, Los Angeles, Calif. All labeled compounds used as substrates were diluted with unlabeled substrate so that the final specific activity was 0.1 to 1.0 μc. per μmole.

Practical grade 2,4-D (Aldrich Chemical Co., Milwaukee, Wis.) was washed twice with methylene chloride prior to use in the culture medium. Analytical grade 2,4-D (Eastman Organic Chemicals) was the substrate employed for enzyme assays. 2,4-Dichlorophenetole, 2,4-dichloroanisole, phenoxyethanol, phenoxyacetic acid, and semicarbazide-HCl were obtained from Eastman Organic Chemicals.

RESULTS

Loss of Methylene- and Carboxyl-Carbon. Four different preparations were incubated with 1 μmole of 2,4-D containing 0.1 μc. of ^{14}C in the carboxyl or methylene carbons of the acetate side chain. The volume of the enzyme preparation, the length of time it was stored after its preparation, and the incubation time varied, however. The amount of radioactivity lost as volatile products and the quantity of chloride formed after incubation with the preparations are shown in Table I. The labeled methylene-carbon was not metabolized by any of the preparations to a product volatilized from the acidified incubation mixture, but the labeled carboxyl-carbon was converted to such a product. The percentage of the carboxyl-label lost was correlated with the amount of chloride released.

Since chloride is freed enzymatically only after cleavage of the ether linkage (Bollag *et al.*, 1968) and the extracts used herein released all the chlorine when incubated with 2,4-dichlorophenol, the per cent of chloride released can be taken as an indication of the extent of ether cleavage. Thus, using chloride release to reflect the occurrence of ether cleavage, it is apparent that for every two molecules of the alkyl moiety released following cleavage of the ether bond, essentially only one of the carboxyl-carbons was lost as a volatile product (Table I). The only discrepancy from the 2 to 1 ratio between percentage of substrate-chlorine released and per cent of carboxyl-carbon lost was preparation C, but in this instance the chloride release was complete.

Identification of Product Derived from Side Chain. At the end of the incubation period, the reaction mixture containing preparation C was examined in an attempt to identify the labeled product. Thin-layer chromatography showed that all of the 2,4-D had been metabolized. The labeled products originating from both carboxyl-labeled and methylene-labeled 2,4-D were not extracted into ethyl acetate from the tungstic acid-treated incubation mixture. Aliquots of the original solutions were then spotted directly onto sheets for thin-layer chromatography. Cellulose sheets were developed in two solvent systems, water-saturated *n*-butanol–98% formic acid (20 to 1) and *n*-butanol–water–acetic acid (10:5:2). In both instances, the metabolites generated from the carboxyl- and methylene-labeled substrates exhibited the same R_f values, and no other sections of the chromatograms contained labeled products. The labeled metabolites had R_f values different from those of glycolic, oxalic, malic, succinic, or citric acids.

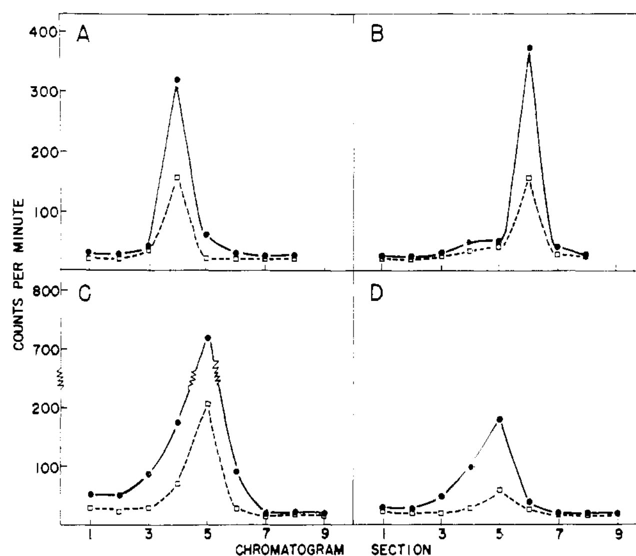

Figure 1. Thin-layer chromatograms of products formed from methylene-labeled (solid line) and carboxyl-labeled 2,4-D (broken line) after development in (A) *n*-butanol–acetic acid–water (4:1:1), (B) chloroform-methanol-NH_4OH-water (20:20:5:4), (C) *n*-propanol–34% NH_4OH (84:34) and (D) *n*-propanol–34% NH_4OH followed by treatment with ninhydrin

Cochromatography of the tagged products with authentic amino acids was carried out on silica gel sheets. The chromatographic location of the label derived from the carboxyl- and methylene-tagged 2,4-D is shown in Figure 1. Only a single radioactive spot was discernible with each of the two substrates, and the two spots had similar R_f values. In the three solvent systems used, the radioactive peak was at the same location as authentic α-alanine. In at least one of the solvent systems, the radioactivity was at a different location on the chromatograms than glutamic acid, aspartic acid, glycine, serine, and γ-aminobutyric acid.

The effect of ninhydrin on the labeled products is also shown in Figure 1. A chromatogram containing replicate samples derived from reaction mixtures incubated with carboxyl- and methylene-labeled 2,4-D was developed and then divided into two portions. One half only was saturated with ninhydrin spray. Both halves were then sectioned in the same way and counted. After treatment with ninhydrin, 78% of the label had volatilized from the spot derived from the reaction mixture incubated with methylene-labeled 2,4-D, whereas 81% had volatilized from the spot derived from the reaction mixture incubated with carboxyl-labeled 2,4-D. Thus, similar quantities of the metabolites were converted to volatile compounds. The expected products of the ninhydrin oxidation of alanine are CO_2 and acetaldehyde, both volatile at room temperature. Although the loss of the carboxyl- and methylene-label should have been complete if the sole product was alanine, the reaction with ninhydrin may not have been stoichiometric.

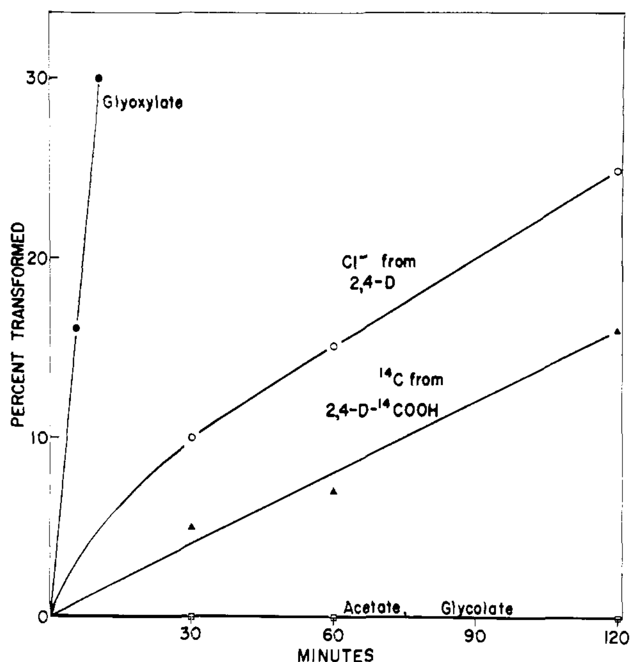

Figure 2. Metabolism of 2,4-D and several aliphatic acids by soluble enzyme preparation. Acetate metabolism was evaluated by measuring loss of radioactivity

Metabolism of Aliphatic Acids. To compare the metabolism of two-carbon acids with 2,4-D, an amount of preparation D containing 18 mg. of protein was incubated in a final volume of 0.6 ml., with 1 μmole of substrate containing approximately 0.1 μc. of label supplied as acetate-1-^{14}C, acetate-2-^{14}C, glycolate-2-^{14}C, 2,4-D acetic acid-1-^{14}C, and 2,4-D acetic acid-2-^{14}C. Loss of label from the reaction mixtures occurred when 2,4-D acetic acid-1-^{14}C was the substrate, but not when the other tagged compounds were supplied as potential substrates (Figure 2). Colorimetric analysis revealed that, although glycolate did not disappear, glyoxylate was metabolized at a rate much faster than 2,4-D. Herbicide degradation in this instance was assessed by measuring the rate of chloride release and volatilization of the tagged carboxyl-carbon.

Colorimetric assays revealed that glycolate was metabolized by a different enzyme preparation prepared in an identical manner. This preparation contained 21 mg. of protein, and it was made and tested immediately after the culture was harvested.

If glyoxylate is formed from the side-chain of 2,4-D, a loss of one-half the carboxyl-carbon might be expected in light of the previous findings. To determine whether this occurred, an amount of the enzyme preparation containing 10 mg. of protein was incubated for 3 hours in 1.0 ml. of solution, having 1 μmole of glyoxylate containing 1 μc. of glyoxylate-1-^{14}C. Colorimetric analysis showed that all the glyoxylate had disappeared by the end of the incubation period. Moreover, analysis of the solution after the incubation mixture was acidified showed that 45% of the initial radioactivity had been lost.

Removal of Low-Molecular Weight Components. To test the effect of removal of low-molecular-weight components on the activity of the enzyme preparation on glyoxylate, a preparation containing 16 mg. of protein was incubated with 1 μmole of substrate. In a 35-minute incubation period, all the glyoxylate had disappeared. When this preparation was passed through a column of Sephadex G-50 to remove small molecules, the same amount of protein catalyzed the disappearance of only 15% of the substrate in the 35-minute period.

Because the activity on glyoxylate was diminished by gel filtration, it was deemed possible that a preparation treated in the same way might retain its activity on 2,4-D while losing much of the activity on glyoxylate, thereby allowing the presumed intermediate to accumulate. To test this possibility, a preparation active on 2,4-D was passed through Sephadex G-50 and then incubated separately with 1 μmole of carboxyl- and methylene-labeled 2,4-D for 3 hours. None of the methylene-carbon and only 9% of the carboxyl-carbon was converted to volatile products in the incubation period. The major labeled compound present on thin-layer chromatograms prepared from the reaction mixture was residual 2,4-D in a concentration nearly equal to that originally supplied. Colorimetric analyses revealed that glyoxylate had not accumulated in detectable levels. Moreover, using fractions obtained from a Sephadex G-150 column (Loos *et al.*, 1967a) which brought about the accumulation of 0.1 μmole of a phenol when incubated with 1 μmole of 4-chloro-2-methylphenoxyacetate—i.e., fractions in which ether-cleaving activity was demonstrated—glyoxylate in quantities detectable by the colorimetric method was still not observed.

Semicarbazide Trap for Glyoxylate. Semicarbazide has been used as a trap for the glyoxylate formed in certain enzymatic reactions (Kornberg and Gotto, 1961; Olson, 1959). The pH of the incubation mixture must be near 6.0 for the semicarbazide to serve as an effective trap. However, when the crude cell extract was incubated at pH 6.3 with carboxyl-labeled 2,4-D in the presence of 10 μmoles of freshly neutralized semicarbazide, the ether cleavage reaction was inhibited by 71% as measured by volatilization of the carboxyl-carbon. When 27 mg. of resting cells was incubated at pH 6.0 with 5 μmoles of carboxyl-labeled 2,4-D in the presence of 100 μmoles of freshly neutralized semicarbazide, the loss of the ^{14}C-carboxyl-carbon was inhibited by 42%. When 1 μmole of oxythiamine was added to the above incubation mixture, the inhibition was 44%. The antimetabolite was used in an attempt to inhibit a possible condensation of glyoxylate catalyzed by a thiamine pyrophosphate-requiring carboxyligase. Nevertheless, a limited amount of ether cleavage had occurred with both the extract and the resting cells. Hence, 50 μmoles of synthetic glyoxylate semicarbazone was added to the supernatant after precipitation of the protein of the extract or removal of the cells by centrifugation. The aqueous supernatant was reacted with 2,4-dinitrophenylhydrazine in 2*N* HCl for 1 hour, extracted with ethyl acetate, and examined by thin-layer chromatography for the presence of radioactive products. No radioactivity was found in the chromatogram sections containing glyoxylate 2,4-dinitrophenylhydrazone. The only labeled compound apparently present was unmetabolized 2,4-D.

Metabolism of Phenoxyacetates by Resting Cells. To determine the substrate specificity of the bacterial ether cleavage, resting cells were incubated with various phenoxy compounds. Two micromoles of substrate was incubated with 170 to 200 mg. (dry wt.) of cells in a 5.0-ml. volume. The activity was measured as the amount of chloride released from the chlorinated substrates or the quantity of phenol formed from phenoxyacetate and phenoxyethanol. In 1 hour, all of the phenoxyacetate and 90% of the 2,4-D had been metabolized. Although no activity was noted after 1 hour,

68% of the phenoxyethanol and 51% of the 2-(2,4-dichlorophenoxy)propionate had been metabolized in 20 hours. No activity was noted in a 20-hour incubation period with 2,4-dichloroanisole, 2,4-dichlorophenetole, 3-(2,4-dichlorophenoxy)propionate, 2- and 4-(2,4-dichlorophenoxy)butyrates, and 3-(2,4-dichlorophenoxy)isobutyrate as substrates. Thus, the *Arthrobacter* sp. shows a high degree of substrate specificity.

If phenoxyethanol was cleaved to yield phenol and an aldehyde, glycolaldehyde would be the expected product. To determine whether such a compound accumulated, 21 mg. of resting cells was incubated with 1 μmole of either phenoxyacetate or phenoxyethanol for 5 hours. The results showed that phenoxyacetate was converted stoichiometrically to phenol, but the phenol formed from phenoxyethanol was only 16% of theoretical. No glycolaldehyde was detected in either incubation mixture by colorimetric analysis. Moreover, gas chromatographic analysis showed only unchanged phenoxyethanol in ether extracts of the phenoxyethanol incubation mixture.

When the crude cell extract was incubated for 5 hours with 1 μmole of phenoxyacetate, the phenol recovery was again stoichiometric. However, phenol was not found in a parallel incubation mixture containing phenoxyethanol as substrate.

DISCUSSION

The *Arthrobacter* sp. employed in the present investigation cleaves the ether bond of several phenoxyacetates to yield the corresponding phenols (Loos *et al.*, 1967a). Although glyoxylate has not been isolated as a reaction product, the fate of the carboxyl- and methylene-carbons of 2,4-D suggests that glyoxylate is indeed formed from the acetate moiety of 2,4-D. Of the likely two-carbon compounds—acetate, glycolate, glyoxylate, and oxalate—that could be formed following the ether cleavage, acetate and glycolate can be eliminated, inasmuch as neither is metabolized under conditions suitable for the degradation of the 2,4-D side chain. Although slight activity was noted on glycolate in one instance, no radioactive product chromatographically similar to glycolate was observed. Oxalate can also be eliminated because randomization of the methylene- and carboxyl-labels, expected if oxalate was an intermediate, did not occur.

Glyoxylate, by contrast, is acted upon more rapidly than 2,4-D by the enzyme preparation, and about one-half of the carboxyl-carbon is lost from the glyoxylate during the incubation. A similar quantity of carbon is lost from the carboxyl-group of 2,4-D when the quantity of herbicide transformed enzymatically, measured by chloride release from the aromatic products generated after ether cleavage, was considered. The inability to demonstrate glyoxylate accumulation probably results from the fact that the rate of its oxidation exceeded the rate of its formation.

All of the methylene-carbon and one-half of the carboxyl-carbon appeared as a single nonvolatile product. The nonvolatile radioactive product was identified as alanine by chromatography in three solvent systems and its susceptibility to volatilization following ninhydrin treatment. The alanine is probably formed from glyoxylate by a pathway involving a condensation of two two-carbon compounds.

The glycerate pathway first described by Kornberg and Gotto (1961) and Krakow *et al.* (1961), the glycine-serine pathway described by Sagers *et al.* (1961), and the β-hydroxyaspartate pathway of Kornberg and Morris (1965) involve the condensation of two glyoxylate or glycine molecules to produce alanine, with a loss of one of the two carboxyls. The glycerate pathway appears to be the most widely encountered of the three and is a likely pathway for the formation of alanine from the side-chain of 2,4-D.

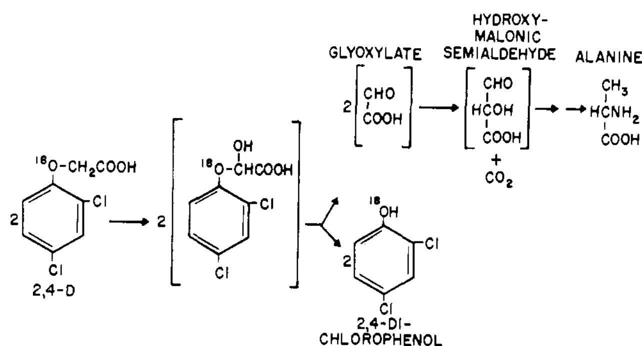

Figure 3. Proposed pathway for ether cleavage and subsequent metabolism of acetate moiety of 2,4-D

The glyoxylate presumably formed and the phenol produced are in accord with a metabolic pathway that involves an initial oxidation of the methylene-carbon to form an α-hydroxy compound (Figure 3). The latter is presumably cleaved to yield 2,4-dichlorophenol and glyoxylate. The glyoxylate may then be converted to α-alanine via hydroxymalonic semialdehyde. The fate of the ether-oxygen, as shown in Figure 3 by the ^{18}O, has been established by the studies of Helling *et al.* (1968).

Monooxygenases cleaving aryl-alkyl ether bonds were first demonstrated by Axelrod (1956), who found that rabbit liver microsomes catalyzed the cleavage of *p*-ethoxyacetanilide to yield *p*-hydroxyacetanilide and acetaldehyde. The reaction required O_2 and reduced nicotinamide adenine dinucleotide phosphate. More recently, Cartwright and Smith (1967) and Cartwright and Buswell (1967) reported that a partially purified bacterial demethylase cleaved vanillate to yield protocatechuate and formaldehyde. Reduced pyridine nucleotides are required in that reaction.

The cofactor requirements of the proposed ether-cleavage sequence have not been adequately demonstrated. Helling *et al.* (1968) reported that O_2 was required. The ether cleavage reaction may require 0.5 or 1.0 mole of O_2 per mole of substrate, depending upon whether the second atom of O_2 is reduced to H_2O or is used to oxidize a second mole of substrate. A requirement for a reduced pyridine nucleotide has not been demonstrated, although the lability of the preparation (complete loss of activity in 4 days) may result from the instability of reduced cofactors in the preparation.

The proposed oxidative sequence differs from the cleavage mechanism suggested by MacRae and Alexander (1963), who identified fatty acids as the products formed from phenoxyalkanoic acids with long fatty acid side-chains. The *Flavobacterium* sp., however, was not active on 2,4-D.

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LITERATURE CITED

Axelrod, J., *Biochem. J.* **63**, 634 (1956).
Bergmann, J. G., Sanik, J., Jr., *Anal. Chem.* **29**, 241 (1957).
Bollag, J.-M., Briggs, G. G., Dawson, J. E., Alexander, M., J. Agr. Food Chem. **16**, 829 (1968).

Bray, G. A., *Anal. Biochem.* **1,** 279 (1960).
Cartwright, N. J., Buswell, J. A., *Biochem. J.* **105,** 767 (1967).
Cartwright, N. J., Smith, A. R. W., *Biochem. J.* **102,** 826 (1967).
Coe, E. L., *Anal. Biochem.* **10,** 236 (1965).
Gaunt, J. K., Evans, W. C., *Biochem. J.* **79,** 25P (1961).
Helling, C. S., Bollag, J.-M., Dawson, J. E., J. AGR. FOOD CHEM. **16,** 538 (1968).
Kornberg, H. L., Gotto, A. M., *Biochem. J.* **78,** 69 (1961).
Kornberg, H. L., Morris, J. G., *Biochem. J.* **95,** 577 (1965).
Krakow, G., Barkulis, S. S., Hayashi, J. A., *J. Bacteriol.* **81,** 509 (1961).
Loos, M. A., Bollag, J.-M., Alexander, M., J. AGR. FOOD CHEM. **15,** 858 (1967a).
Loos, M. A., Roberts, R. N., Alexander, M., *Can. J. Microbiol.* **13,** 691 (1967b).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Lyle, S. J., Sani, A. R., *Anal. Chim. Acta* **33,** 619 (1965).
MacRae, I. C., Alexander, M., *J. Bacteriol.* **86,** 1231 (1963).
Olson, J. A., *J. Biol. Chem.* **234,** 5 (1959).
Sagers, R. D., Benziman, M., Gunsalus, I. C., *J. Bacteriol.* **82,** 233 (1961).
Trijbels, F., Vogels, G. D., *Biochim. Biophys. Acta* **113,** 292 (1966).

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