Phenoxyacetate Herbicide Detoxication by Bacterial Enzymes

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Extracts of an *Arthrobacter* sp. contained enzymes dehalogenating 2,4-dichloro-, 2-methyl-4-chloro-, and 2- and 4-chlorophenoxyacetates. From the phenoxyacetates, these enzyme preparations produced compounds with the chromatographic characteristics of the corresponding phenols. The bacterial extracts further metabolized the phenols as well as several chloro- and methylcatechols. The enzyme acting on the phenoxy compounds was separated from the enzyme metabolizing the chlorophenols.

The phenoxyacetates are one of the major groups of herbicides used for selective control of weeks, and the vast amounts that are used annually are degraded ultimately to yield nontoxic products. Despite the significance of microorganisms for the detoxication of pesticides in the environment, little is known of the microbial mechanisms of degradation. Hydroxylation of the aromatic ring has been reported to occur in microorganisms as well as in plants (Evans and Smith, 1954; Faulkner and Woodcock, 1961; Thomas *et al.*, 1964), and evidence has been obtained which indicates that certain bacteria are capable of removing the acetate moiety following ring hydroxylation (Evans and Smith, 1954; Gaunt and Evans, 1961). By contrast, the present authors' work with intact cells of an *Arthrobacter* sp. indicated that the side chain of 2,4-dichlorophenoxyacetate (2,4-D) was removed without prior hydroxylation of the benzene ring (Loos *et al.*, 1967a, b).

Detailed studies of the mechanisms of breakdown of the chlorinated phenoxyacetate herbicides have been hampered by the lack of an enzyme preparation able to decompose these compounds. The preparation and some properties of a bacterial enzyme system which catalyzes the degradation of 2,4-D, 2- and 4-chlorophenoxyacetates (2-CPA and 4-CPA), and 2-methyl-4-chlorophenoxyacetate (MCPA) are here described.

MATERIALS AND METHODS

The phenoxyacetate-degrading *Arthrobacter* sp. described previously (Loos *et al.*, 1967a) was cultured with aeration at 25° C. in the following medium: 2,4-D or MCPA (neutralized with NaOH), 3.0 grams as the free acid; KH_2PO_4, 1.2 grams; K_2HPO_4, 4.8 grams; NH_4NO_3, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; $CaCl_2 \cdot 2H_2O$, 25 mg.; $FeCl_3 \cdot 6H_2O$, 2.5 mg.; and distilled water, 1.0 liter. The bacteria were collected by centrifugation, the cells were washed twice with 0.01*M* phosphate buffer, pH 7.2, and resuspended in the same buffer. The cell suspension was passed twice through a cooled French pressure cell operated at 20,000-p.s.i. pressure. Whole cells and the larger components of broken cells were removed by centrifuging the suspension at 20,000 × G for 30 minutes to yield the crude extract. The soluble fraction was obtained by centrifuging the crude extract at 144,000 × G for 1 hour.

Solutions containing 0.5 ml. of extract (14.7 to 23.0 mg. of protein) 0.5 ml. of 0.01*M* phosphate buffer, pH 7.2, and 1.0 μmole of substrate were incubated with shaking at either 30° or 25° C. The reactions were stopped, and protein was precipitated by adding 10 ml. of the tungstic acid reagent of Van Slyke and Hawkins (Hawk *et al.*, 1954). The tungstic acid-treated reaction mixtures were analyzed for chloride using the method of Bergmann and Sanik (1957). Corrections were made for chloride introduced with the extract, in substrate solutions, and in the tungstic acid solution. No chloride was released from any of the substrates, in the absence of extract, by the addition of tungstic acid. Metabolism of phenol or catechol was assessed by measuring the extent of disappearance of the substrates (Lacoste *et al.*, 1959; Mitchell, 1924). In the catechol assay, 0.05% osmic acid was added to a solution buffered at pH 7.8.

RESULTS AND DISCUSSION

Both the crude extract and the soluble fraction derived from it metabolized 2,4-D with the release of 92 and 80% of the organic chlorine, respectively, in a 3-hour incubation period. Extracts of 2,4-D- or MCPA-grown cells also produced chloride when incubated with other phenoxyacetates as well as with several phenols and catechols (Table I). Enzymes in the preparation apparently are capable of metabolizing several phenoxyacetate herbicides, including not only 2,4-D but also MCPA and 4-CPA. The failure of the extract to dehalogenate 6-hydroxy-2,4-dichlorophenoxyacetate suggests that it is not an intermediate in the degradation of 2,4-D by this microorganism, in contrast with the findings of Evans and Smith (1954) with pseudomonads.

Phenols and catechols were also metabolized by the crude bacterial extracts, as indicated by phenol or catechol disappearance (Table I). Activity was noted with 2,4-dichlorophenol and 2-methyl-4-chlorophenol, the compounds which would be formed upon cleavage of the ether linkage of 2,4-D and MCPA, respectively, as well as with 2- and 4-chlorophenol. By measuring the rate of chloride formation at regular intervals, it was shown that the rate of dehalogenation of the chlorophenols was essentially the same whether the extracts were prepared from cells cultured on 2,4-D or MCPA. The 4-chloro- and the 3,5-dichlorocatechols, possible subsequent intermediates in the degradation of the herbicides, were also enzymatically dechlorinated. During the course of the reaction, the phenol or catechol was destroyed. The extent of degradation was similar whether chloride release or phenol or catechol disappearance was used to assay enzymatic action.

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Table I. Metabolism of Phenoxyacetates, Phenols, and Catechols by Extracts of an *Arthrobacter* sp.

Substrate	Incubation Time, Hours	Chloride Release, %	Substrate Disappearance, %
2,4-Dichlorophenoxyacetate	3	92[a]	
2-Methyl-4-chlorophenoxyacetate	4	39	
2-Chlorophenoxyacetate	4	72[a]	
4-Chlorophenoxyacetate	4	70[a]	
6-Hydroxy-2,4-dichlorophenoxyacetate	3	0[a]	
2-Hydroxy-4-chlorophenoxyacetate	3	28[a]	
2,4-Dichlorophenol	4	100[a]	
2-Methyl-4-chlorophenol	4	72	65
2-Chlorophenol	4	65	82
4-Chlorophenol	4	68	80
4-Chlorocatechol	4	77[a]	63
3,5-Dichlorocatechol	4	90[a]	
3-Methylcatechol	4		84
4-Methylcatechol	4		81
Catechol	4		100

[a] Extract prepared from 2,4-D-grown cells and incubated at 30° C. In all other instances, extracts were prepared from MCPA-grown cells and incubated at 25° C.

With the exception of 3,5-dichlorocatechol, from which 16% of the bound chlorine was liberated, no halide was formed when these chlorine-containing phenoxyacetates, phenols, and catechols were incubated for 4 hours with an extract heated for 5 minutes at 80° C.

If the first step in 2,4-D decomposition is a decarboxylation, the product would be 2,4-dichloroanisole. To determine whether the anisole is an intermediate in the degradation, 0.5 ml. of the crude extract prepared from 2,4-D grown cells was incubated with 0.5 ml. of 0.01*M* phosphate buffer, pH 7.2, and approximately 2 μmoles of 2,4-dichloroanisole. Although some 2,4-dichloroanisole remained undissolved throughout the incubation period, gas chromatographic analysis showed the presence in the aqueous solution of small amounts of the substrate (less than 10 μg. per ml.). The amount of chloride detected in the reaction mixture at the end of a 3-hour incubation period was equivalent to only about 7% of the bound chlorine supplied. The inability of the extract to metabolize 2,4-dichloroanisole at a significant rate, if at all, suggests that it is not an intermediate in 2,4-D breakdown by the bacterium.

Phenolic intermediates were produced by the soluble fraction during the degradation of 2,4-D, 4-CPA, and 2-CPA. The reaction mixture contained 11.2 mg. of extract-protein when 2,4-D was the substrate or 12.4 mg. of extract-protein when 4-CPA or 2-CPA was provided. At regular intervals, tungstic acid was added to individual tubes containing the reaction mixtures, and aliquots were extracted with ether. The ether extract was dried with anhydrous Na_2SO_4, concentrated where necessary, and chromatographed using an Aerograph Model A-700 gas chromatograph fitted with a flame ionization detector. The column was a coiled glass tube, 10 feet long × 3/8-inch I.D., containing DC 200 silicone oil (12,500 centistokes) on 80- to 100-mesh, acid-washed Chromosorb W, in a 1 to 9 ratio by weight. N_2 (flow rate, 190 ml. per minute) was the carrier gas. The operating temperatures were: column, 200°; injector, 250°; and detector, 260° C. Compounds with retention times of 187, 116, and 170 seconds had accumulated after incubation of the soluble fraction with 2,4-D, 2-CPA, and 4-CPA. These retention times are identical to those of authentic 2,4-dichlorophenol and 2- and 4-chlorophenol, respectively (Figure 1).

The soluble fraction was concentrated by lyophilization, and the concentrated preparation was placed on a column of Sephadex G-150 equilibrated with 0.02*M* phosphate buffer, pH 7.0. The column was eluted with the same buffer, and activity of the fractions which were collected was tested using MCPA, 2-methyl-4-chlorophenol, and 4-chlorocatechol as substrates. No activity on 2-methyl-4-chlorophenol, as measured by phenol destruction or chloride release, was found in any of the fractions, but the enzyme (or enzymes) catalyzing the conversion of MCPA to a phenol was demonstrable in certain fractions. This enzyme did not release halide from MCPA. By contrast, chloride was liberated by preparations containing the enzyme acting upon 4-chlorocatechol (Figure 2). Thus, dehalogenation of MCPA apparently occurs after removal of the aliphatic moiety from the molecule.

Because the eluate from the Sephadex column was devoid of phenol-metabolizing activity, it was possible to isolate and characterize the phenol generated enzymatically from MCPA. The phenol was collected from the reaction mixture and identified on the basis of its retention time in the gas chromatograph as 2-methyl-4-chlorophenol.

The phenoxyacetate-cleaving enzyme of this bacterium is labile, and its activity is lost after several days. The reaction appears to be enzymatic, inasmuch as activity is abolished upon heating at 80° C. for 5 minutes. Gas chromatographic analysis showed that solutions containing either the enzyme preparations or the substrates alone did not contain compounds corresponding to the metabolites observed.

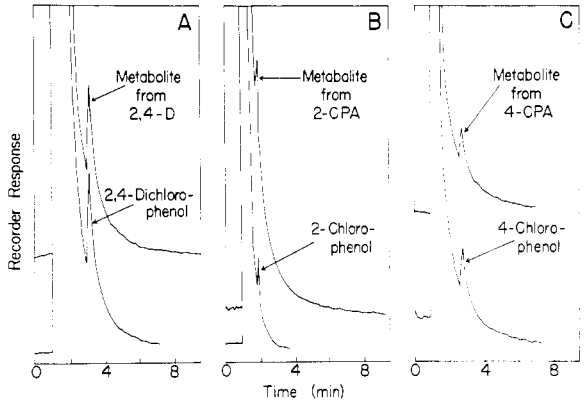

Figure 1. Gas chromatograms of authentic chlorophenols and metabolites

The metabolites were produced by the soluble fraction after incubation with 2,4-D for 1 hour (*A*) and with 2-CPA (*B*) and 4-CPA (*C*) for 2 hours. The volume of sample injected into the chromatograph was 15, 5, and 5 μl., respectively

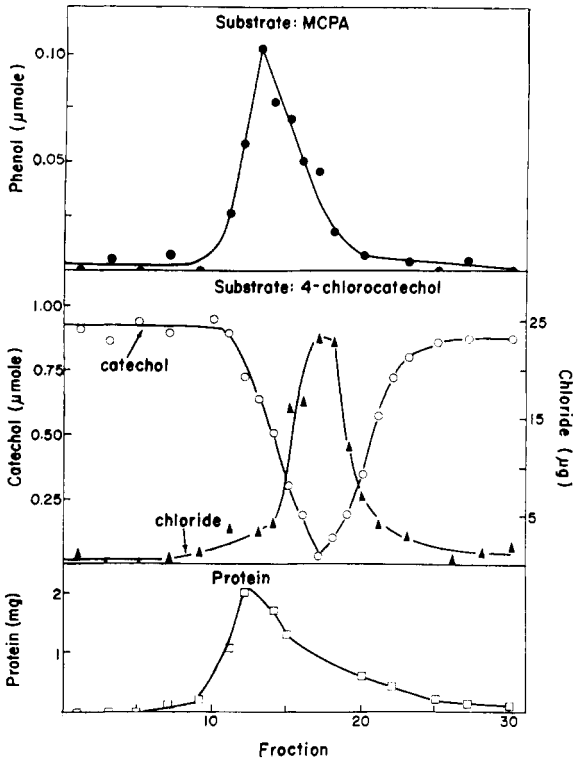

Figure 2. Enzymatic activity of Sephadex G-150 eluate on MCPA and 4-chlorocatechol

Fractions of 3.5 ml. were collected, and the reactions were measured using 1.0 μmole of substrate and 1.0 ml. of the eluate. Values for protein represent protein content of the fraction

Intact cells of the *Arthrobacter* sp. convert phenoxyacetates to their corresponding phenols (Loos *et al.*, 1967b). The finding that enzymes in extracts of this bacterium convert 2,4-D, 2-CPA, 4-CPA, and MCPA to the corresponding phenols and also further degrade these compounds not only shows unequivocally the intermediary role of phenols in the biological decomposition but also makes it possible to study the individual enzymatic steps in the metabolism of phenoxyacetate herbicides.

The conversion of phenoxyacetates to phenols has been reported to occur in plants (Chkanikov *et al.*, 1956). The elucidation of the enzymatic mechanism involved in degradation of phenoxyacetates and phenols by the *Arthrobacter* sp. should provide a basis for understanding how plant tissues as well as microorganisms act upon these compounds.

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