2,4-D Metabolism

Enzymatic Hydroxylation of Chlorinated Phenols

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A chlorophenoxyacetate-degrading *Arthrobacter* sp. contained an enzyme which converted 2,4-dichlorophenol and other chlorophenols to catechols. Reduced nicotinamide adenine dinucleotide phosphate and O_2 were required in the reaction. The enzyme hydroxylating 2,4-dichlorophenol was partially separated from the catechol-degrading enzyme present in the bacterial extract. The compounds formed from 2,4-dichlorophenol and 4-chlorophenol were identified as 3,5-dichlorocatechol and 4-chlorocatechol, respectively.

Microbial activity is responsible, at least in part, for the degradation of phenoxyalkanoate herbicides that are applied to or enter a variety of natural habitats. Although large amounts of such pesticides are used each year, the products formed as a result of the microbial conversions and the biochemical mechanisms by which the phenoxyalkanoates are mineralized are inadequately known.

Intact cells of a soil bacterium, a strain of *Arthrobacter*, are capable of converting 2,4-dichlorophenoxyacetate (2,4-D) and other phenoxyacetates to the corresponding phenols. The 2,4-dichlorophenol that is generated from 2,4-D and a number of other chlorophenols are in turn oxidized by the microbial cells (Loos *et al.*, 1967b,c). From this microorganism, an enzyme preparation has been obtained which cleaves the ether linkage between the phenolic and fatty acid moieties of the phenoxyalkanoate substrate to yield the free phenol; thus, 2,4-D, 4-chloro-2-methyl-, and 2- and 4-chlorophenoxyacetates are converted enzymatically to products with the chromatographic characteristics of 2,4-dichlorophenol, 4-chloro-2-methylphenol, and 2- and 4-chlorophenol, respectively (Loos *et al.*, 1967a). The present report is concerned with the metabolism of the chlorinated phenols generated by the phenoxyacetate-detoxifying enzyme system. In the following paper (Bollag *et al.*, 1968), data on the subsequent fate of the original 2,4-D molecule are presented.

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MATERIALS AND METHODS

The phenoxyacetate-metabolizing *Arthrobacter* sp. was cultured in a medium containing 4-chloro-2-methylphenoxyacetate (MCPA), as previously described (Loos *et al.*, 1967a), and the cells were harvested during the exponential growth phase. The cell paste was washed three times in $0.02M$ phosphate buffer, pH 7.0, homogenized in two volumes of the same buffer, and then passed through a French pressure cell operated at 15,000-p.s.i. pressure. Whole cells and the larger components of broken cells were removed by centrifugation at $10,000 \times G$ for 10 minutes, and the resulting supernatant was centrifuged at $144,000 \times G$ for 60 minutes. The clear yellow supernatant was decanted and lyophilized. The dried material could be stored at $-20°$ C. for several months without appreciable loss of activity. Aliquots of the lyophilized extracts were dissolved in aqueous solution as needed.

Enzyme activity was determined at 25° C. in solutions containing 20 μmoles of phosphate buffer, pH 7.0, and 1.0 μmole of substrate. The incubation was terminated after 6 hours by addition of the tungstic acid reagent of Van Slyke and Hawkins (Hawk *et al.*, 1954), and the proteins were removed by centrifugation. Anaerobic activity was assessed in Thunberg tubes from which the air was removed and replaced with N_2 (99.9% purity). The soluble enzyme preparation was fractionated by placing 25 mg. of the lyophilized extract contained in 0.3 ml. of water on a 2.0×45.0 cm. column of Sephadex gel (Pharmacia Fine Chemicals, Inc., New Market, N. J.) and eluting the enzymes with $0.02M$ phosphate buffer, pH 7.0, at an average flow rate of 180 drops per hour. Fractions containing 3.5 ml. were collected.

Protein was measured by the Folin phenol method (Lowry *et al.*, 1951) with crystalline egg albumin as standard. Phenols were determined by a modification of the

4-aminoantipyrine procedure (Loos *et al.*, 1967b) using 2,4-dichlorophenol as standard, and catechol and chloride concentrations were determined colorimetrically as previously described (Loos *et al.*, 1967a). When the accumulation of a catechol was noted, the fractions containing the compound were pooled for characterization of the unknown. To identify the catechol, the acidified incubation mixture was extracted with an equal volume of diethyl ether, which was then dried with anhydrous Na_2SO_4 and treated under nitrogen purge with a 20% (w./v.) lead acetate solution (Helling and Bollag, 1968). The insoluble lead complex which formed was washed twice with water, dried at 105° C., and examined as a KBr micropellet by infrared spectroscopy. Paper microframes containing 2 × 10 mm. apertures were used with a beam condenser. Infrared spectra were obtained with a Beckman spectrophotometer, Model IR10.

For gas chromatography, the Aerograph chromatograph (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.), Model A-700, was employed (Loos *et al.*, 1967a). To achieve minimal tailing and maximal response for the catechols, the column was primed immediately before use by repeated injections of standard catechols.

The phenols and catechols, obtained from Eastman Organic Chemicals, Rochester, N.Y., were vacuum-sublimed before use. Flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and reduced nicotinamide adenine dinucleotide (NADH) were obtained from the Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Although the extract of the bacterium is capable of metabolizing several substituted phenols, the eluate from the column after passage of the soluble fraction through Sephadex G-150 showed no activity on several chlorinated phenols. Several cofactors were added to the protein-containing fractions in an attempt to restore the phenol-metabolizing activity. Upon the addition of reduced pyridine nucleotides, chlorophenol oxidation was readily demonstrable. The data in Table I show that 2,4-dichlorophenol disappeared and a catechol was generated if NADPH or NADH was present, the former coenzyme having an appreciably greater effect on the reaction. The addition of FAD or ferrous ions did not enhance the activity, and quinacrine was not inhibitory. Up to 90% of the phenol metabolized was recovered as the catechol. A ferrous ion requirement could not be demonstrated even after dialysis of these fractions against phosphate buffer. No activity was found if air was excluded from the reaction mixture. The requirement for both oxygen and NADPH suggests that the activity is catalyzed by a mixed-function oxidase. An NADPH requirement for the oxidation of the 4-chloro-2-methylphenol generated enzymatically from MCPA has been reported for the *Arthrobacter* sp. (Bollag *et al.*, 1967).

Those fractions from the column which metabolized 2,4-dichlorophenol also acted upon catechol, 4-chlorocatechol, 3,5-dichlorocatechol, and 3- and 4-methylcatechols. To separate the catalyst acting on 2,4-dichlorophenol from the enzyme or enzymes degrading the catechols, the lyophilized extract was chromatographed on a Sephadex G-200 column. As shown in Figure 1, a partial separation of these proteins was achieved, the enzyme metabolizing 2,4-dichlorophenol being eluted prior to the catalyst concerned in 4-chlorocatechol degradation. In those fractions in which a separation was obtained, the accumulation of a catechol-like compound generated from the chlorinated phenols was noted. Thin-layer chromatography of the reaction mixture on silica gel F_{254} (E. Merck AG, Darmstadt, Germany) with benzene–dioxane–acetic acid (90:25:4) as solvent suggested that the metabolite generated was indeed a catechol, but it was not possible by this chromatographic method to distinguish among the individual catechols.

For identification of the product formed from 2,4-dichlorophenol, a lead derivative of the metabolite was prepared. The infrared spectrum of the lead complex of the catechol generated enzymatically from 2,4-dichlorophenol coincided with that of authentic lead 3,5-dichlorocatecholate (Figure 2). The spectrum showed a major band at ca. 1390 and minor ones at 675 and 1040 cm.$^{-1}$

Table I. Cofactor Requirements for Enzymatic Hydroxylation of 2,4-Dichlorophenol

Addition[a]	Phenol Disappearance, μmole	Catechol Formation, μmole
None	0.00	0.00
NADH	0.28	0.15
NADPH	0.78	0.66
Fe^{2+}	0.00	0.00
FAD	0.00	0.00
NADPH + Fe^{2+}	0.74	0.59
NADPH + FAD	0.76	0.69
NADPH + quinacrine	0.75	0.67
NADPH (anaerobic)	0.04	0.04

[a] One μmole added, except that 20 mμmoles of FAD was used. Each tube contained 0.35 mg. of protein.

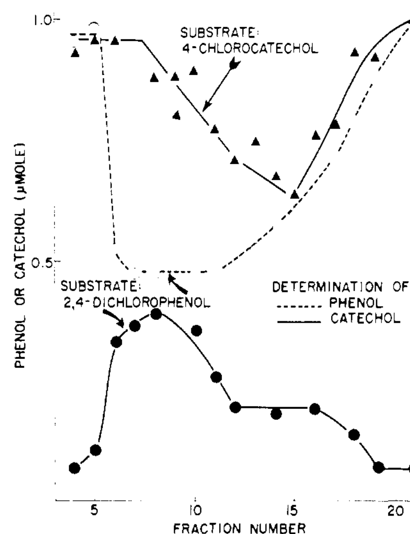

Figure 1. Fractionation of *Arthrobacter* sp. enzymes metabolizing 2,4-dichlorophenol and 4-chlorocatechol

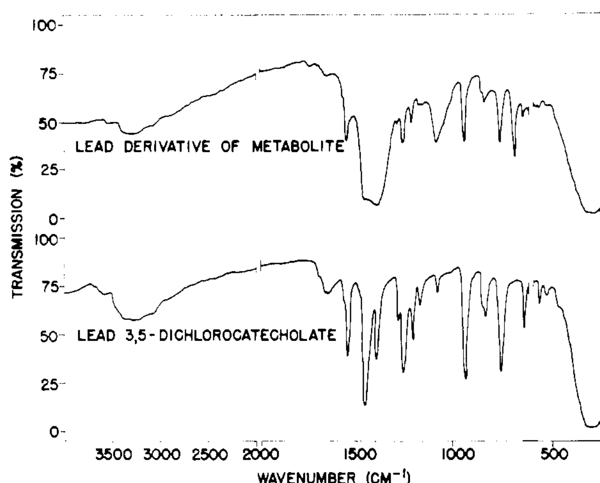

Figure 2. Infrared spectra of lead 3,5-dichlorocatecholate and lead derivative of a metabolite formed from 2,4-dichlorophenol

arising from contaminating basic lead carbonate. The free catechol was liberated from the lead complex by suspending 9.1 mg. of the salt in 1.0 ml. of water and treating the suspension with 0.5 ml. of 22% (w./v.) aqueous $(NH_4)_2S$. Gas chromatographic analysis of the catechol thus liberated provided further evidence of its identity with 3,5-dichlorocatechol.

The catalysts metabolizing 4-chloro-2-methylphenol and the chlorocatechols were also readily separated by means of the Sephadex G-200 column. Fractions 8, 9, and 10 contained the 4-chloro-2-methylphenol–oxidizing activity but were devoid of activity on 4-chlorocatechol, as measured by catechol destruction. In these tubes, the enzyme preparation generated appreciable amounts of a catechol from the phenol (Figure 3). No catechol accumulation was observed in subsequent fractions, because the catalyst for catechol metabolism was present as well as the phenol-oxidizing enzyme.

Hydroxylation was likewise demonstrable when 4-chlorophenol, the product of the degradation of 4-chlorophenoxyacetate (Bollag *et al.*, 1967; Loos *et al.*, 1967a), was incubated with fractions of the Sephadex G-200 eluate which were devoid of activity on the catechols. Thus, from 1.0 μmole of 2,4-dichlorophenol, 4-chlorophenol, and 4-chloro-2-methylphenol, 0.74, 0.74, and 0.78 μmole, respectively, of a catechol was produced. In these catechol assays, 4-chlorocatechol was used as the standard compound. Phenol itself was apparently not oxidized, indicating that the enzyme was specific for certain substituted phenols. The lead derivative of the compound produced from 4-chlorophenol had an infrared spectrum identical to that of authentic lead 4-chlorocatecholate, demonstrating that this chlorophenol is also hydroxylated to yield the corresponding catechol prior to cleavage of the aromatic ring. The catechol generated from 4-chloro-2-methylphenol was not identified.

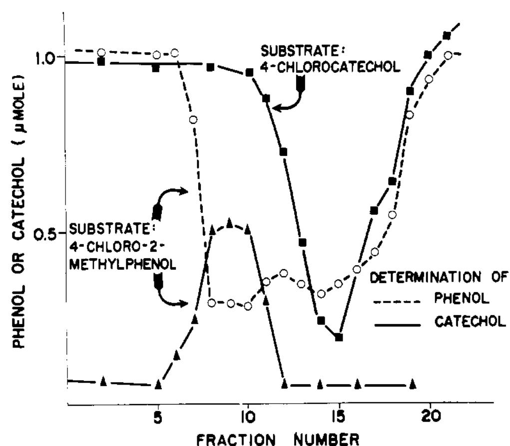

Figure 3. Fractionation of enzymes metabolizing 4-chloro-2-methylphenol and 4-chlorocatechol

The formation of 4-chlorocatechol and 3,5-dichlorocatechol in cultures of a bacterium grown upon 4-chlorophenoxyacetate and 2,4-D was demonstrated by Evans and Smith (1954) and Evans *et al.* (1961). The gram-negative bacterium they investigated also was reported to produce 2-hydroxy-4-chlorophenoxyacetate from 4-chlorophenoxyacetate and 6-hydroxy-2,4-dichlorophenoxyacetate from 2,4-D; evidently the aromatic ring is hydroxylated by this microorganism prior to removal of the fatty acid moiety from the phenoxyacetate molecule. By contrast, enzymatic studies with the *Arthrobacter* sp. show that the substituted phenoxyacetate is not hydroxylated, but rather that the molecule is cleaved to yield the corresponding phenol (Bollag *et al.*, 1967; Loos *et al.*, 1967). The phenol is then the substrate for the hydroxylating enzyme. The halogen is not removed from the aromatic ring prior to catechol formation.

In the following paper (Bollag *et al.*, 1968), the subsequent enzymatic degradation of the catechols formed by the herbicide-degrading microorganism and the products of the decomposition are demonstrated.

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