2,4-Dichlorophenoxyacetate Metabolism by
Arthrobacter sp.: Accumulation of a
Chlorobutenolide

K. W. SHARPEE, J. M. DUXBURY, AND M. ALEXANDER
Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14850

Received for publication 18 April 1973

An enzyme preparation from 2,4-dichlorophenoxyacetate-grown Arthrobacter sp. converted cis, cis-2, 4-dichloromuconate to chloromalaylacetate. The enzyme lactonizing the dichloromuconate to yield 2-chloro-4-carboxymethylene but-2-enolide was separated from the butenolide-lactonizing enzyme.

Decomposition of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) by Arthrobacter sp. involves cleavage of the ether bond to yield 2,4-dichlorophenol, oxidation of the phenol to the corresponding catechol, cleavage of the aromatic ring to form cis, cis-2, 4-dichloromuconate, and then a conversion of the muconate to succinate with the intermediary formation of a chloromalaylacetate (3, 5). The probable intermediate between cis, cis-2, 4-dichloromuconate and the chloromalaylacetate is 2-chloro-4-carboxymethylene but-2-enolide. Although this lactone is metabolized by extracts of Arthrobacter sp., its pivotal role in 2,4-D metabolism by this bacterium remains obscure, because of its lack of accumulation when enzyme preparations are incubated with potential butenolide precursors generated during 2,4-D decomposition (5).

The present study was designed to determine whether the chlorobutenolide is an intermediate in 2,4-D metabolism by Arthrobacter sp.

 Cultures of Arthrobacter sp. were grown at 25 C in a 0.2% 2, 4-D-mineral salts medium (5). The cells were collected by centrifugation and washed three times with cold 0.02 M Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.1, containing 0.001 M MnSO₄. This buffer was also used in the subsequent disruption and fractionation procedures. The cells were suspended in 80 ml of buffer containing 2 ml of glass beads (20–30 μm diameter) and 0.1 mg of deoxyribonuclease, and they were disrupted with a sonic oscillator. The resulting preparation was centrifuged at 55,000 × g for 1 h to yield the crude extract.

The cis, cis-2, 4-dichloromuconate-lactonizing enzyme was assayed by measuring the change in absorbance at 268 nm, which is the wavelength of maximum absorbance for the dichloromuconate. The assay was performed at 25 C in quartz cuvettes containing 3.0 ml of 0.02 M Tris-hydrochloride buffer, pH 7.1, 0.10 μmol of cis, cis-2, 4-dichloromuconate, 0.70 μmol of MnSO₄, and enzyme. The 2-chloro-4-carboxymethylene but-2-enolide-lactonizing enzyme was assayed at 25 C by measuring the change in absorbance at 283 nm, the wavelength of maximum absorbance of the chlorobutenolide, in quartz cuvettes containing 3.0 ml of 0.02 M potassium phosphate, pH 8.0, 0.1 μmol of the chlorobutenolide, and enzyme. The chlorobutenolide was synthesized from 3,5-dichlorocatechol, previously purified by vacuum sublimation, by periodate oxidation. A unit of enzyme was defined as the amount necessary to cause the disappearance of 1 μmol of substrate per min under the conditions of the assay.

To synthesize the cis, cis-2, 4-dichloromuconate used in enzyme assays, 0.2 to 0.4 mg of a 40 to 50% saturated (NH₄)₂SO₄ fraction was added to 20 μmol of 3, 5-dichlorocatechol contained in 100 ml of 0.02 M Tris-hydrochloride buffer, pH 7.1. This reaction mixture was incubated at 25 C until no further increase in absorbance at 268 nm occurred. The reaction was then terminated by adding an equal volume of acetone, and the solution was centrifuged at 27,000 × g for 10 min to remove the protein precipitate. The acetone was subsequently removed with a flash evaporator maintained at 30 C. The supernatant fluid was extracted twice with ether to remove residual 3, 5-dichlorocatechol, and traces of the ether were then removed by use of a flash evaporator maintained at 30 C. The
amount of dichloromuconate in the solution was determined by adding 4 N HCl to a sample of the liquid until a pH of 1 was attained. The absorbance at 283 nm was then measured, and the concentration of the chlorobutenolide formed by the acidification was determined by reference to a standard curve.

The crude extracts formed dichloromuconate in greatest amounts after about 60 min when 2.2 μmol of 3,5-dichlorocatechol was incubated with 1.0 mg of the extract in 20 ml of 0.02 M Tris-hydrochloride buffer, pH 7.1. The accumulated dichloromuconate was then converted to the chloromaleylacetate within 30 min when 20 μmol of MnSO₄ was added. The same conversion took 1 h in the presence of 20 μmol of MgSO₄. No chloromaleylacetate was generated from the accumulated dichloromuconate, however, when MnSO₄ was added to a reaction mixture containing potassium phosphate instead of Tris buffer. Therefore, Tris buffer and MnSO₄ were used in the assay of the lactonizing enzyme.

A rapid loss of lactonizing enzyme activity occurred when attempts were made to separate it from the lactonizing enzyme by (NH₄)₂SO₄ fractionation in the presence of 0.02 M Tris-hydrochloride buffer (pH 7.1), but this activity was stabilized if the buffer contained 0.001 M MnSO₄. Little separation of the lactonizing and delactonizing enzymes was achieved with (NH₄)₂SO₄ fractionation, however. Most of the lactonizing enzyme was present in the 50 to 60% saturated (NH₄)₂SO₄ fraction, whereas the delactonizing enzyme was mainly in the 40 and 50% fraction. The activities of the lactonizing enzyme present in the crude extract and in the various (NH₄)₂SO₄ fractions were much lower than those for the delactonizing enzyme.

Two milliliters of the 50 to 60% fraction (containing 40 mg of protein) was then placed on a diethylaminoethyl (DEAE)-cellulose column (2.5 by 40 cm) which had been prepared and equilibrated essentially by the procedures of Peterson and Sober (4). The column was eluted with 0.02 M Tris-hydrochloride buffer, pH 7.1, containing 0.001 M MnSO₄ and 0.1 M NaCl, and fractions of 4 ml were collected and analyzed. Successful separation of the two enzymes was achieved by this procedure. The delactonizing enzyme was eluted first from the DEAE-cellulose column in activities much greater than those of the lactonizing enzyme, which was eluted later (Fig. 1).

Fractions 180 to 230 from the column contained only the lactonizing enzyme, and the chlorobutenolide accumulated in these fractions when incubated in the usual assay procedure. The ultraviolet spectra of the chlorobutenolide appearing in these later fractions were identical to that of the chemically synthesized chlorobutenolide, and they exhibited absorption maxima at 283 nm. The lactonizing enzyme in these fractions was not stable, and all of its

![Fig. 1. Separation of the lactonizing and delactonizing enzymes of Arthrobacter sp. by chromatography on DEAE-cellulose.](http://aem.asm.org/Downloaded from http://aem.asm.org/ on May 8, 2020 by guest)
activity was lost within a few hours after elution from the column.

On the basis of the observation that the chlorobutenolide is a substrate for crude extracts of *Arthrobacter* sp. grown on 2,4-D (5), and the present finding that the chlorobutenolide is generated by a partially purified dichloromuconate-lactonizing enzyme obtained from the bacterium, it appears that this compound is an intermediate in the pathway of degradation of 2,4-D by the microorganism. Evans et al. (1) recently presented evidence for the biosynthesis of the chlorobutenolide by a pseudomonad grown on 2,4-D. The chlorobutenolide is of special interest because it contains an unsaturated lactone ring, and compounds of this type are known to have a variety of effects on physiological activities (2).

This investigation was supported by Public Health Service training grant ES-00098 from the Division of Environmental Health Sciences.

LITERATURE CITED
1. Evans W. C., B. S. W. Smith, H. N. Fernley, and J. I. Davies. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. Biochem. J. 122:543-551.
2. Haynes, L. J. 1955. Lactones, p. 583-594. In K. Paech and M. V. Tracey (ed.), Modern methods of plant analysis, vol. 2. Springer-Verlag, Berlin.
3. Loos, M. A. 1969. Phenoxyalkanoic acids, p. 1-49. In P. C. Kearney and D. D. Kaufman (ed.), Degradation of herbicides. Marcel Dekker, New York.
4. Peterson, E. A., and H. A. Sober. 1962. Column chromatography of proteins: substituted cellulosics, p. 3-27. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
5. Tiedje, J. M., J. M. Duxbury, M. Alexander, and J. E. Dawson. 1969. 2,4-D metabolism: pathway of degradation of chlorocatechols by *Arthrobacter* sp. J. Agr. Food Chem. 17:1021-1026.