2,4-D Metabolism

Enzymatic Degradation of Chlorocatechols

J.-M. Bollag,[1] G. G. Briggs,[2] J. E. Dawson, and Martin Alexander

Extracts of an *Arthrobacter* sp., a bacterium which forms 3,5-dichlorocatechol and 4-chlorocatechol from 2,4-dichloro- and 4-chlorophenoxyacetates, metabolized the two catechols with the formation of an ultraviolet-absorbing compound and chloride. At low enzyme levels, the chlorocatechols were metabolized completely, but little chloride was formed. O_2 was required in the reaction, but pyridine nucleotides and iron were not stimulatory. An enzyme preparation was obtained which liberated all the chlorine in 4-chlorocatechol but a maximum of half the chlorine in 3,5-dichlorocatechol. Two compounds found in the reaction mixture after incubation of the crude enzyme preparation with 3,5-dichlorocatechol were characterized by mass spectrometry and infrared spectroscopy, the data indicating one of these to be α-chloro-γ-carboxymethylene-Δ$^{\alpha}$-butenolide. The analytical data suggest that a product generated from 4-chlorocatechol was γ-carboxymethylene-Δ$^{\alpha}$-butenolide.

In the metabolism of the chlorophenoxyacetate herbicides by a soil *Arthrobacter* sp., the parent pesticidal molecule is cleaved enzymatically to yield the corresponding phenol. Thus, 2,4-dichlorophenoxyacetate (2,4-D), 4-chlorophenoxyacetate, and 4-chloro-2-methylphenoxyacetate (MCPA) are converted to 2,4-dichlorophenol, 4-chlorophenol, and 4-chloro-2-methylphenol (Loos *et al.*, 1967). In the preceding paper (Bollag *et al.*, 1968), it was shown that the substituted phenols are then converted to catechols, apparently by a mixed function oxidase. The catechol generated from 2,4-dichlorophenol was identified as 3,5-dichlorocatechol, while that formed from 4-chlorophenol was shown to be 4-chlorocatechol.

The present communication reports an investigation of the fate of the catechols produced microbiologically from the phenoxyacetate herbicides.

MATERIALS AND METHODS

The bacterium, an *Arthrobacter* sp. which is able to use several chlorinated phenoxyacetates as sole carbon source for growth, was cultured with aeration at 25° C. in 18 liters of the MCPA medium previously described (Bollag *et al.*, 1967). The cultures were harvested during the exponential phase of growth by centrifugation at 4° C. The cells were washed three times in 0.02*M* phosphate buffer, pH 7.0; the paste was homogenized in two volumes of the same buffer, and then passed twice through a cooled French pressure cell operated at 15,000-p.s.i. pressure. Intact cells and cell debris were removed by centrifugation for 10 minutes at approximately 10,000 × G. These procedures were carried out at a temperature not exceeding 5° C. The cell extract was centrifuged at 25,000 × G for 100 minutes and the resulting supernatant lyophilized. The lyophilized cell extract retained its chlorocatechol-metabolizing activity for several months.

To demonstrate enzyme activity, the lyophilized extract was dissolved in water, and 0.5 ml. of this preparation or a fraction derived from it was incubated with shaking at 30° C. in 1.1 ml. of a reaction mixture containing 10 μmoles of phosphate, pH 7.0, and 1.0 μmole of substrate. The reaction was terminated by the addition of 0.083*N* H_2SO_4 solution containing 10% $Na_2WO_4 \cdot 2H_2O$, the proteins being removed by centrifugation. A boiled enzyme control was included in all assays. For anaerobic incubations, Thunberg tubes were evacuated, and N_2 (99.9% pure) was flushed through the tubes. Protein, catechol, and chloride determinations were performed by the procedures outlined (Bollag *et al.*, 1967).

To detect products of catechol metabolism, the acidified protein-free reaction mixtures were extracted with an equal volume of diethyl ether, the ether extracts were dried with anhydrous Na_2SO_4, the solvent was removed by evaporation, and the residue was redissolved in distilled water. The formation of catechol-degradation products in the reaction mixtures was then measured at 260 mμ in a Beckman spectrophotometer, Model DU. The ether extract was also examined for possible intermediates by means of an Aerograph Model A-700 gas chromatograph equipped with a flame ionization detector. The glass column employed was 3.0 meters × 7.5-mm. i.d. and contained 10% DC 200 methylsilicone oil (12,500 centistokes) on 80- to 100-mesh Gas-Chrom Q obtained from the Applied Science Laboratories. The operating temperatures for the column, injector, and detector were 180°, 200°, and 250° C., respectively. The flow rate of the carrier gas, N_2, was 150 ml. per minute. The products were converted to their trimethylsilyl derivatives by treatment of the ether extract with bis-(trimethylsilyl)acetamide (BSA) at room temperature.

Mass spectra were obtained with an Associated Electrical Industries (Manchester, England) mass spectrometer, Model MS 9, using a direct inlet system. Infrared spectra of KBr micropellets were determined with a Beckman

Department of Agronomy, Cornell University, Ithaca, N. Y. 14850

[1] Present address, Department of Agronomy, The Pennsylvania State University, University Park, Pa.

[2] Present address, Chemistry Department, Rothamsted Experimental Station, Harpenden, Herts, England

spectrophotometer, Model IR10, equipped with beam condenser.

Protocatechuic acid was recrystallized twice from water. All other catechols used as substrates were purified by vacuum sublimation. *trans,trans*-Muconic acid (*trans*, *trans*-2,4-hexadienedioic acid) was obtained from the Baker Chemical Co., Phillipsburg, N. J. α,α'-Dimethyl-*cis,cis*-muconic acid was a gift from the Sun Oil Co., Marcus Hook, Pa. γ-Carboxymethyl-Δ^{α}-butenolide was prepared by the method of Elvidge *et al.* (1950).

RESULTS

The bacterial cell extract metabolized 4-chlorocatechol and 3,5-dichlorocatechol, as indicated by measurements of catechol disappearance, chloride formation, or changes in absorbancy at 260 mμ in the residue from ether extract. Typical results with 4-chlorocatechol as substrate are shown in Figure 1. Under optimum conditions, 0.5 ml. of crude extracts containing 2.5 mg. of protein destroyed 1.0 μmole of substrate in 1 to 2 hours. The observed chloride release may be enzymatic or may result from the nonenzymatic cleavage of an unstable chlorinated intermediate.

By contrast with other ring-cleaving enzymes (Kita, 1965; Kojima *et al.*, 1961), the chlorocatechol-metabolizing enzyme lost little activity when stored in the presence of air. No ring cleavage was noted when the enzyme was incubated anaerobically with the catechols. The addition of 1.0 μmole of ferrous iron or of oxidized or reduced nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate did not increase the rate of catechol degradation by these extracts. The apparent absence of a cofactor requirement is also suggested by the finding that the proteins of the extract still retained catechol-decomposing activity when separated from lower molecular weight components of the preparation by fractionation on Sephadex G-200 or, as reported by Loos *et al.* (1967), on Sephadex G-150.

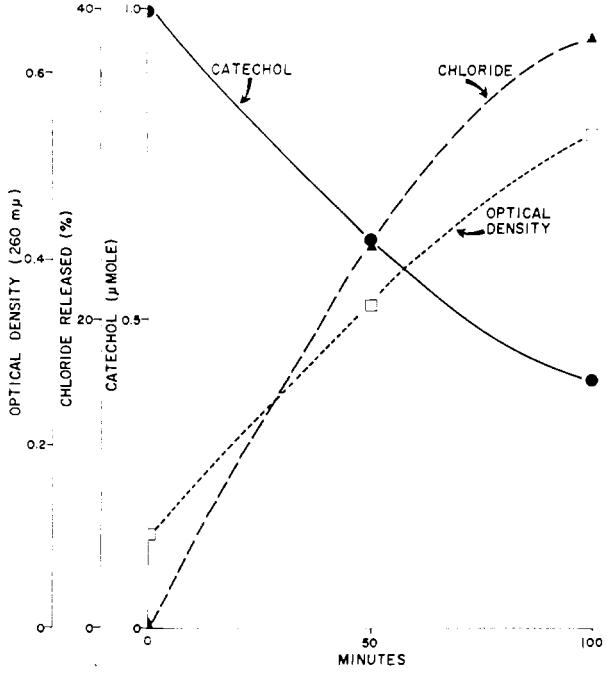

Figure 1. Metabolism of 4-chlorocatechol by *Arthrobacter* sp. extracts

At high concentrations of 4-chlorocatechol, the amount of chloride formed was reduced. Thus, when the bacterial extract containing 16.5 mg. of protein per ml. was incubated with 1.0, 2.0, and 5.0 μmoles of 4-chlorocatechol for 60 minutes at 30° C., 85, 34.5, and 9.0% of the bound chlorine, respectively, were converted to free chloride.

Because of the large amount of ultraviolet-absorbing materials in the original extract, ultraviolet measurements failed to reveal whether a muconic acid or a muconolactone was formed from the chlorocatechol. However, the formation of a product or products absorbing at 260 mμ was readily demonstrated when dilutions of the extract were incubated with 4-chlorocatechol. Thus, in a reaction mixture containing 0.62 mg. of protein per ml., the absorbancy at 260 mμ increased with time as the 4-chlorocatechol was decomposed.

The accumulation of the product or products absorbing at 260 mμ was dependent upon the concentration of the crude extract employed in the assay. At low enzyme levels, all the 4-chlorocatechol was degraded in the incubation period, and the unknown ultraviolet-absorbing material had accumulated; however, little chloride was liberated (Figure 2). This suggests that the dehalogenation step follows enzymatic destruction of the catechol. The data are also consistent with the hypothesis that the unknown intermediate still retains the chlorine. The quantity of ultraviolet-absorbing material decreased, while the amount of chloride formed in extracts incubated with 4-chlorocatechol increased with increasing concentrations of the extracts, presumably resulting from the dehalogenation of a chlorinated intermediate. In these assays, colorimetric evidence for complete catechol disappearance was confirmed by gas chromatography.

When various dilutions of the extract were incubated with 3,5-dichlorocatechol, complete substrate disappearance in the 4-hour incubation period also occurred at low extract concentrations, and again incomplete chloride liberation was noted in the more dilute enzyme solutions (Figure 2). In this instance, the maximum absorbancy at 260 mμ was less than when 4-chlorocatechol was the substrate.

Colorimetric measurements of catechol disappearance indicated that the enzyme preparation was also active on catechol and 3- and 4-methylcatechols but not on protocatechuic acid. When an amount of the extract containing 2.0 mg. of protein was incubated with 1.0 μmole of catechol or 3-methylcatechol, absorption at 260 mμ increased with time. In a comparative study of three substrates, the accumulation of ultraviolet-absorbing material was faster with catechol and 3-methylcatechol than with 4-chlorocatechol. The enzyme preparation did not catalyze the release of chloride from chlorohydroquinone, 4-chlororesorcinol, and 2- and 3-chlorobenzoates.

Twenty-five milligrams of the lyophilized preparation was dissolved in 0.5 ml. of water and applied to a refrigerated column (35.0 × 2.0 cm.) of Sepharose 2B. The column was eluted with 0.02*M* phosphate buffer, pH 7.0, and 3.5-ml. fractions were collected. The activity of the eluate was tested by incubating 1.0 ml. of the various fractions for 6 hours with 1.0 μmole of 4-chlorocatechol

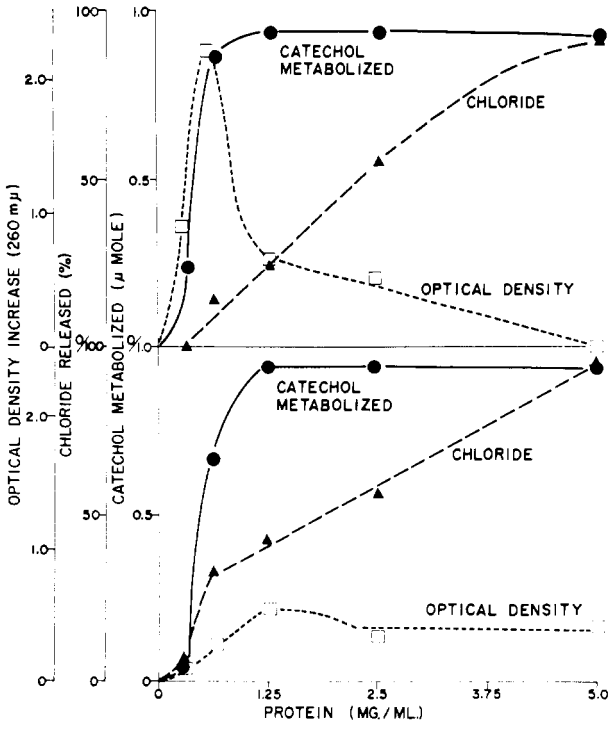

Figure 2. Effect of enzyme concentration on metabolism of 4-chlorocatechol (top) and 3,5-dichlorocatechol (bottom)

Incubation period 3 hours

or 3,5-dichlorocatechol at 30° C. Colorimetric analysis revealed that essentially all the 4-chloro- and 3,5-dichlorocatechols had disappeared in fractions 23 to 29 (Figure 3). Ether extracts of those tubes in which 4-chloro- or 3,5-dichlorocatechol had been partially or completely destroyed showed the accumulation of an intermediate(s) absorbing at 260 mμ. Of particular interest is the fact that, although the enzymes in certain fractions released all the chlorine in 4-chlorocatechol, a maximum of half of the chlorine in 3,5-dichlorocatechol was converted to the ionic form. Thus, apparently two dehalogenases are involved in the degradation of 3,5-dichlorocatechol. The data also suggest the conversion of 3,5-dichlorocatechol to an intermediate containing one chlorine and the conversion of 4-chlorocatechol to a nonchlorinated product, presumably the chlorine on carbon-5 of dichlorocatechol being removed first.

A second fractionation was performed and gave results essentially identical to those depicted in Figure 3. Gas chromatography of these reaction mixtures after incubation with 3,5-dichlorocatechol showed no catechol but rather a product with a retention time similar to γ-carboxymethyl-Δ^{α}-butenolide in fractions 23 to 26, unchanged catechol and a second compound in fractions 27 and 28, and the substrate as well as the apparent carboxymethylbutenolide in fractions 30 to 36.

To isolate sufficient product for identification, 100 μmoles of 3,5-dichlorocatechol or 4-chlorocatechol and

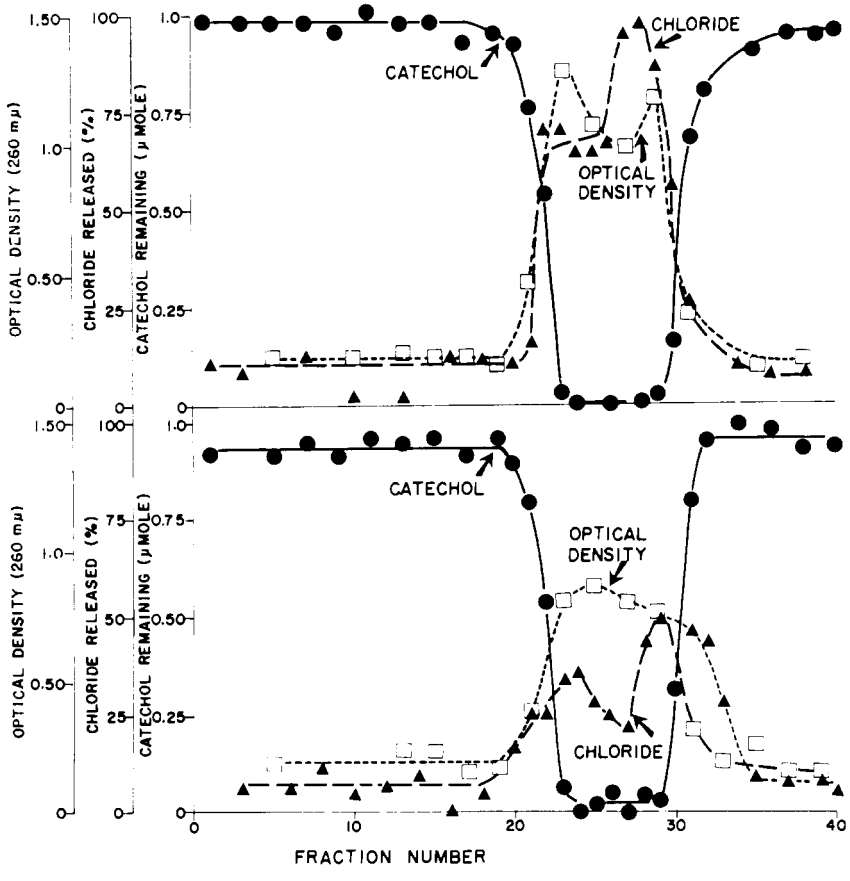

Figure 3. Gel filtration of bacterial extract active on 4-chlorocatechol (top) and 3,5-dichlorocatechol (bottom)

the extract (50 mg. of protein) contained in 100 ml. of 0.02*M* potassium phosphate buffer, pH 7.0, were incubated at 30° C. on a rotary shaker for 3 hours. After precipitation of the proteins with the tungstic acid reagent, the supernatant of the reaction mixture was extracted twice with an equal volume of ether. The water in the ether extract was removed by anhydrous Na_2SO_4, and the solution was evaporated to dryness. Infrared spectra of the material extracted from both reaction mixtures were similar, but not identical, in the 1600- to 2000-cm.$^{-1}$ region of the spectrum. Strong peaks were observed at 1800, 1785, 1695, and 1655 cm.$^{-1}$ for the product from 3,5-dichlorocatechol and at 1795, 1760, 1690, and 1650 cm.$^{-1}$ for the product from 4-chlorocatechol. Neither catechol absorbs in the region above 1600 cm.$^{-1}$ The absorption near 1800 cm.$^{-1}$ suggests that an unsaturated lactone was present (Bellamy, 1958).

Compounds in the extract were converted to their trimethylsilyl derivatives. Examination of these derivatives in the gas chromatograph indicated that large amounts of unchanged catechol still remained. The gas chromatograms also showed two major products formed from 3,5-dichlorocatechol. Neither appeared to be a muconic acid, as suggested by comparisons of their retention times with those of standard *trans,trans-* or *cis,cis*-muconic acids or of α,α'-dimethyl-*cis,cis-* or β-chloromuconic acid. The compound in the extract which had a retention time similar to that of authentic γ-carboxymethylbutenolide was collected from the chromatograph exit port in a trap cooled in ice water. The infrared spectrum of this product showed major peaks at 1800, 1695, and 1650 cm.$^{-1}$, indicating that it was an unsaturated lactone.

Low resolution mass spectra were obtained for the two ether extracts. The volatile catechols dominated the mass spectral traces obtained immediately after insertion of the probe, but the catechol peaks decreased in intensity after a few minutes, and additional components could be observed more clearly. The mass spectra showed that a product with mass 174 had been formed from 3,5-dichlorocatechol. The 3-to-1 ratio between molecular ions of mass 174 and 176 indicated that this product contained a chlorine atom. A chlorine-containing fragment of mass 157 was observed, indicating the loss of a hydroxyl. The probable formula for this compound is $C_6H_3ClO_4$ (Silverstein and Bassler, 1967). An unchlorinated product with mass 140 was formed enzymatically from 4-chlorocatechol, and fragmentation patterns suggested that it too contained a hydroxyl. The probable formula for this compound is $C_6H_4O_4$. Compounds which fit the mass spectral and infrared data are α-chloro-γ-carboxymethylene-Δ^{α}-butenolide from 3,5-dichlorocatechol and γ-carboxymethylene-Δ^{α}-butenolide from 4-chlorocatechol.

In a second experiment carried out under similar conditions, 200 mg. of lyophilized enzyme preparation and 100 μmoles of 3,5-dichlorocatechol were employed. The dried ether extract obtained after the termination of the incubation period showed a major absorption band at 1760 cm.$^{-1}$ and a less intense absorption at 1620 cm.$^{-1}$, in addition to bands attributable to unmetabolized dichlorocatechol. Treatment of the crude product with petroleum ether (boiling point 60° to 80° C.) removed the catechol, leaving a pale brown powder which contained one major component detectable by gas chromatography. High and low resolution mass spectra were obtained on this material. There was a 3-to-1 ratio between molecular ions of mass 148 and 150 in the low resolution spectrum, showing the presence of chlorine in the molecule. Fragmentation ions of masses 133 and 105 were noted, suggesting the loss of CH_3 and of CH_3CO. The chlorine was not readily lost, indicating its attachment to a double bond.

From the high resolution mass spectrum of this compound, a molecular weight of 147.9929 was obtained. After subtracting the atomic weight of chlorine-35 from this value and using appropriate tables, the molecular formula was $C_5H_5ClO_3$. CO_2 was not lost from the molecule in the mass spectrometer, and this fact together with the infrared spectrum (Figure 4) indicates that the molecule contains no free carboxyl group.

DISCUSSION

The initial steps in the pathway by which the phenoxyacetate herbicides are degraded by *Arthrobacter* sp. involve the formation of the corresponding phenol from the parent compound by cleavage of the ether linkage, the phenol in turn being enzymatically hydroxylated in the ortho position to yield the halogenated catechol (Bollag *et al.*, 1968; Loos *et al.*, 1967). In the present study, evidence is presented that both 3,5-dichlorocatechol and 4-chlorocatechol are metabolized by an O_2-requiring enzyme to yield an intermediate which is not a catechol but still retains the halogens of the catechol substrate. In a subsequent reaction, one of the chlorines from the dichloro compound is released in ionic form, a reaction which may be catalyzed by the same enzyme which dehalogenates 4-chlorocatechol. The data thus indicate the sequential formation of a dichloro- and a monochloro-containing intermediate from 3,5-dichlorocatechol and a monochloro and a nonhalogenated intermediate from 4-chlorocatechol. Inasmuch as the crude extract catalyzes the conversion of both halogens of the dichlorocatechol to chloride and the growing culture releases essentially all the chlorine of 2,4-D, a nonhalogenated product is formed from the dichlorocatechol as well.

Mass spectrometry established that a compound with the probable formula $C_6H_3ClO_4$ was generated from 3,5-dichlorocatechol and a compound with the probable formula $C_6H_4O_4$ from 4-chlorocatechol. Considering the structures of possible products formed in either of the two clearly established pathways for the ring fission of catechols (Dagley, 1967), only α-chloro-γ-carboxymethylene-Δ^{α}-butenolide or its dilactone would have the correct structure for the products from 3,5-dichlorocatechol. The corresponding unchlorinated compound would appear to be generated from 4-chlorocatechol. Gaunt and Evans (1961) reported the isolation of α-methyl-γ-carboxymethylene-Δ^{α}-butenolide from bacterial cultures incubated with MCPA. The lactonic acid structure is suggested by the peak in the infrared spectrum near 1690 cm.$^{-1}$, indicating an α,β-unsaturated acid, the apparent loss of OH in the mass spectrometer, and the reaction with BSA under conditions in which butyrolactone does not react. These products are consistent with ortho-cleavage of the catechol, followed by lactonization with displacement of chlorine. An ortho-cleavage mechanism would suggest, moreover,

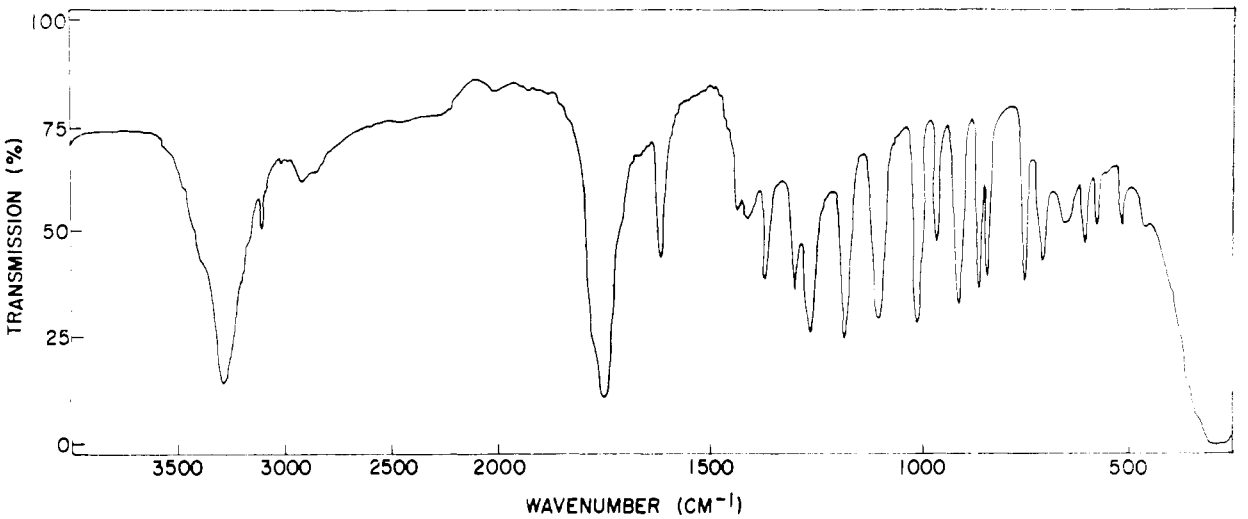

Figure 4. Infrared spectrum of intermediate with formula $C_5H_3ClO_3$

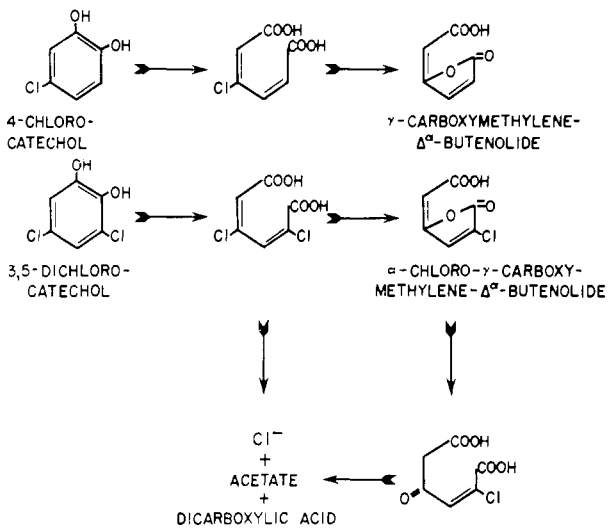

Figure 5. Proposed pathway for degradation of chlorocatechols by *Arthrobacter* sp.

that the nondehalogenated metabolites from 3,5-dichlorocatechol and 4-chlorocatechol, the existence of which is supported by the results presented herein, are probably the α,γ-dichloro- and β-chloromuconic acids, respectively.

The second compound found in enzyme preparations incubated with 3,5-dichlorocatechol had a molecular formula of $C_5H_3ClO_3$ and contained a methyl but no free carboxyl group. A compound arising from the degradation of α-chloro-γ-carboxymethylene-Δ^{α}-butenolide would have a linear arrangement of carbon atoms with chlorine next to the carboxyl group. The carbonyl absorption at 1760 cm.$^{-1}$ indicates a γ-lactone, and the compound is, therefore, probably a derivative of α-chloro-γ-methylbutyrolactone.

The fate of the carboxymethylenebutenolides is not yet known. Considering the evidence presented above favoring the ortho-cleavage mechanism of aromatic metabolism and assuming that the analogy holds for subsequent metabolic steps, one might anticipate the formation of a chlorosubstituted β-ketoadipate. This could be cleaved directly to yield acetate and chloromaleate, reduced and then cleaved to give acetate and chlorosuccinate, or dehalogenated prior to further degradation of the C_6-dicarboxylic acid (Figure 5). The suggestion that β-chloromuconate is a precursor of the butenolide in the metabolism of 4-chlorophenoxyacetate is consistent with the finding of Evans and Moss (1957) that a β-chloromuconate was formed by a gram-negative bacterium grown on this phenoxyacetate. Fernley and Evans (1959) also noted an α-chloromuconic acid formed by a soil pseudomonad from 2,4-D.

The toxicity of these products to higher plants or to other organisms is unknown. Inasmuch as they are found in cultures of a microorganism obtained from soil, they may accumulate during the decomposition of phenoxy herbicides. In this regard, it is of interest that compounds containing unsaturated lactone rings commonly possess some form of physiological activity (Haynes, 1955).

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