2,4-D Metabolism: Pathway of Degradation of Chlorocatechols by *Arthrobacter* sp.

J. M. Tiedje,[1] J. M. Duxbury, Martin Alexander, and J. E. Dawson[2]

Enzymes obtained from an *Arthrobacter* sp. grown on 2,4-dichlorophenoxyacetic acid catalyzed the conversion of 4-chloro- and 3,5-dichlorocatechols to *cis,cis*-3-chloro- and *cis,cis*-2,4-dichloromuconic acids, respectively, by an ortho-fission mechanism. Extracts of the bacterium also converted catechol, 3- and 4-methylcatechols to the corresponding muconic acids. Upon acidification, the chlorinated *cis,cis*-muconic acids either rearranged to more stable isomers or lactonized with displacement of the β-chlorine atom to form 4-carboxymethylene but-2-enolide and 2-chloro-4-carboxymethylene but-2-enolide, respectively. These butenolides and the corresponding chlorinated *cis,cis*-muconic acids and chlorocatechols were converted enzymatically to identical products, which were tentatively identified as maleylacetic and chloromaleylacetic acids, respectively. Ring-labeled 2,4-dichlorophenoxyacetic acid was metabolized by a soluble enzyme preparation to succinic acid.

Chlorinated catechols are intermediates in the degradation of the widely used chlorinated phenoxyacetic acids. Thus, the enzymatic degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) proceeds via 2,4-dichlorophenol to yield 3,5-dichlorocatechol. Similarly, 4-chlorophenoxyacetic acid is metabolized via 4-chlorophenol to 4-chlorocatechol (Bollag *et al.*, 1968b; Loos *et al.*, 1967). Inasmuch as vast land areas and considerable amounts of edible plant materials are treated with such phenoxyacetic acids, identification of the subsequent metabolic products assumes considerable importance in toxicology, environmental monitoring, and ecology.

The pathway of microbial degradation of the chlorocatechols remains somewhat obscure. Reportedly, bacteria generate 3- and 2-chloromuconic acids in the oxidation of 4-chloro- and 2,4-dichlorophenoxyacetic acids, respectively (Evans and Moss, 1957; Fernley and Evans, 1959). The formation of 4-carboxymethylene but-2-enolide, maleylacetic acid, and fumarylacetic acid has also been observed (Evans *et al.*, 1961). Recently, Bollag *et al.* (1968a) reported the isolation of 2-chloro-4-carboxymethylene but-2-enolide from an enzyme preparation incubated with 3,5-dichlorocatechol. Although this compound was not shown to be an intermediate in the degradation, its presence was considered consistent with the operation of an ortho-cleavage pathway for 3,5-dichlorocatechol metabolism.

The present study was designed to determine the products formed during the biological degradation of chlorinated catechols and to establish the pathway by which chlorophenoxyacetic acids and intermediates in their degradation are metabolized.

MATERIALS AND METHODS

Enzyme Preparation. The *Arthrobacter* sp. previously described (Loos *et al.*, 1967) was grown at 25° C. in 40 liters of a medium containing 80 grams of 2,4-D, 14.5 grams of NaOH, 192 grams of K_2HPO_4, 48 grams of KH_2PO_4, 20 grams of NH_4NO_3, 8.5 grams of $MgSO_4 \cdot 7H_2O$, 1.5 grams of $CaCl_2 \cdot 2H_2O$, 0.1 gram of $FeCl_3 \cdot 6H_2O$, 0.04 mg. of $MnCl_2$, and 0.14 mg. of $ZnSO_4$. The final pH was 7.3. A 4-liter inoculum grown for two to three days in the same medium was employed, and air was passed through the medium at a rate of 75 liters per minute. The cells were harvested by centrifugation when the culture was 20 to 30 hours old, and the cells were then washed three times in cold 0.02*M* phosphate buffer, pH 7.3.

The cell pellet was suspended in the same buffer, and the cells were ruptured by means of a French pressure cell operated at 15,000 to 20,000 p.s.i. Whole cells and larger components of broken cells were removed by centrifuging at 20,000 × G for 30 minutes. The remaining particulate components were removed by centrifuging at 140,000 × G for 90 minutes to yield a soluble preparation, which was lyophilized and stored at −10° C. The activity of the soluble extract remained stable for at least three months. In studies in which succinate formation from 2,4-D was demonstrated, the enzyme preparation was made using the X-Press by methods already described (Tiedje and Alexander, 1969).

Incubation Conditions. All substrates were dissolved in 0.02*M* potassium phosphate buffer, pH 7.3, and neutralized with NaOH prior to incubation. Unless otherwise stated, the reaction mixtures were incubated for 3 hours at 28° C. with shaking. The rate of accumulation of the muconic acids and their UV characteristics were determined employing 5.0 ml. of reaction mixture containing 0.3 μmole of catechol and the enzyme preparation (0.3 mg. of protein). To obtain the 3-chloro- and 2,4-dichloromuconic acids designated as "biological," 0.3 μmole of 4-chloro- or 3,5-dichlorocatechol was incubated for 3 hours in a 1.0-ml. volume with 2.0 mg. of an appropriate protein fraction obtained from a Sephadex G-200 column. To demonstrate the further metabolism of the muconic acids, the reaction mixture containing the biologically formed muconate or 0.3 μmole of a synthetic muconic acid was incubated with the soluble enzyme preparation (1.0 mg. of protein) in a final volume of 1.1 ml.

To obtain sufficient quantities of muconic acids for isolation and chemical characterization, incubation mixtures containing 48 μmoles of substrate and 24 mg. of protein in 432 ml. of buffer were used. The incubation was stopped and the products extracted when the reaction mixture showed maximum absorbance at 260 mμ. For preparation of the maleylacetic acids, 20 μmoles of 4-chlorocatechol, butenolide, and chlorobutenolide were incubated with 10 mg. of protein for 3 hours in 11 ml. of solution. To obtain chloromaleylacetic acid from 3,5-dichlorocatechol, 20 μmoles of substrate were incubated with 20 mg. of enzyme-protein in 22 ml.; at higher concentrations, this catechol was inhibitory. In studies showing the accumulation of succinate, 1.0 μmole of ring-labeled

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

[1] Present address, Department of Soil Science, Michigan State University, East Lansing, Mich. 48823

[2] Deceased

2,4-D (0.2 μc. per μmole) was incubated with a one-day-old extract (52 mg. of protein) in 1.2 ml. of solution.

Special Chemicals. *cis,cis*-Muconic acid, *cis,cis*-2-methylmuconic acid, *cis,trans*-3-methyl-, and *cis,trans*-3-chloromuconic acid were synthesized by periodate oxidation of the corresponding catechol (Duxbury and Dawson, 1969). Other isomers were prepared by rearrangement of the above mentioned acids using standard procedures (Elvidge *et al.*, 1950; Sugita *et al.*, 1958). Chlorobutenolide, m.p. 134–6° C. (2-chloro-4-carboxymethylene but-2-enolide) and butenolide, m.p. 159–62° C. (4-carboxymethylene but-2-enolide) were synthesized from 3,5-dichlorocatechol and 4-chlorocatechol, respectively (Duxbury and Dawson, 1969).

Maleylacetic acid (*cis*-3-keto-but-1-ene-1,4-dicarboxylic acid) was prepared by hydrolysis of the butenolide overnight at 0° C. in 1*N* aqueous NaOH, and chloromaleylacetic acid (*cis*-1-chloro-3-keto-but-1-ene-1,4-dicarboxylic acid) was made by hydrolysis of the chlorobutenolide for three days at 0° C. in 0.02*M* phosphate buffer, pH 7.3. The maleylacetic acids were isolated, as colorless oils, by continuous ether extraction of the acidified hydrolysis mixture.

Chlorolactol (lactol form of *cis*-2-chloro-4-keto-pent-2-enoic acid) was prepared by heating a solution of the chlorobutenolide in phosphate buffer (pH 7.3) for 30 minutes at 75° C., followed by ether extraction of the acidified reaction mixture. The crude product was recrystallized from benzene-hexane to give colorless needles, m.p. 95–8° C.

The *Arthrobacter* sp. was grown on practical grade 2,4-D which was washed twice with methylene chloride prior to use. 3-Methylcatechol was recrystallized from benzene, and 4-chlorocatechol was purified by vacuum sublimation. The 4-chlorocatechol still contained 6 to 10% catechol, as determined by gas chromatogram peak areas, even after several recrystallizations and sublimations. 3,5-Dichlorocatechol was purified by vacuum sublimation until the sample gave a single homogeneous peak on the gas chromatograph.

Isolation Procedures. To isolate the muconic acids, the incubation was terminated by the addition of 4*N* HCl to a pH of 1.5 to 2.0, and the acidified incubation mixture was extracted twice with equal volumes of diethyl ether. The ether extract was dried with anhydrous Na_2SO_4 and then concentrated under reduced pressure. To isolate the maleylacetic acids, a small amount of Na_2SO_4 was added to aggregate the protein in the acidified solution. The protein was removed by centrifugation, the supernatant was extracted continuously with ether for 2 hours, and the products were isolated as previously described. The products obtained after extraction are designated "isolated" products, whereas those present in the incubation mixture are referred to as "biological products."

Analytical Methods. The radioactive products from ring-labeled 2,4-D were identified by cochromatography on Eastman Chromagram silica gel sheets and on Whatman No. 1 paper. Acidic compounds were detected by a spray of 0.04% bromcresol purple in ethanol. The area of the chromatograms in which radioactive products were expected was cut into approximately 1.5-sq.-cm. sections and the sections placed in a scintillation vial for assay.

Routine thin-layer chromatography was performed on Eastman Chromagram silica gel plastic sheets (with fluorescent indicator) or on plates made up with Merck silica gel, grade 7741. Components were detected by a short wavelength UV light, iodine absorption, or by spraying with 0.04% bromcresol purple in ethanol.

Gas chromatographic analyses were performed with an Aerograph Model 200 gas chromatograph equipped with a flame ionization detector. The column contained 1 to 9 (w/w) DC 200 silicone oil on 60- to 80-mesh Chromosorb W, HMDS treated. The column temperature was 175° C. unless otherwise stated. Samples were chromatographed as the trimethylsilyl (TMS) derivatives, which were prepared using an excess of *N,O*-bis-(trimethylsilyl)acetamide (BSA).

Ultraviolet absorption spectra were determined with a Beckman spectrophotometer, Model DB, usually in 0.02*M* phosphate buffer (pH 7.3) or, in certain specified instances, in 0.02*M* phosphate acidified to pH 2.0. Corrections were made, if necessary, for protein in the incubation mixture. Samples for infrared analysis were pressed into KBr pellets, and the spectra were determined with a Beckman spectrophotometer, Model IR10. Nuclear magnetic resonance spectra were measured on a Varian Associates A60 spectrometer. Chloroform was used as solvent with tetramethyl silane as internal reference. Mass spectra were obtained on 9-μg. samples using an Associated Electrical Industries (Manchester, England) MS 9 mass spectrometer equipped with a direct inlet system; the samples were subjected to a 70 eV. ionization potential.

Catechol disappearance was followed quantitatively by the osmic acid procedure (Loos *et al.*, 1967). Chloride was assayed by the method of Bergmann and Sanik (1957). Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard. The presence of β-ketoadipate was estimated qualitatively by the alkaline nitroprusside procedure (Feigl, 1956).

RESULTS

Catechol Oxidation. Enzymes in the soluble fraction of the *Arthrobacter* sp. rapidly metabolized several catechols. When the enzyme preparation was incubated with catechol, 3-methyl-, 4-methyl-, 4-chloro-, and 3,5-dichlorocatechols, a linear increase in UV absorbance was noted because of the generation of catechol-degradation products. If the product accumulation was measured at the absorption maximum of the corresponding muconic acids (*cis,cis*-muconic, 2-methyl-, 3-methyl-, 3-chloro-, and 2,4-dichloromuconic acids), apparently the two methylcatechols were metabolized at about equal rates and more rapidly than the remaining three substrates. The maximum concentration of the five presumed muconic acids was achieved after incubation periods of 70, 30, 30, 60, and 80 minutes, respectively. In each instance, the quantity of muconic acid accumulated, based upon the extinction coefficients, suggested an almost complete conversion of the catechols to the corresponding muconic acids.

The analytical data obtained for the muconic acids are summarized in Table I. The retention times are those of the TMS derivatives. The infrared spectra and retention times of the crystalline product isolated following the enzymatic oxidation of catechol and 3-methylcatechol were identical with those of authentic *cis,cis*-muconic and *cis,cis*-2-methylmuconic acid, respectively. The product isolated from 3-methylcatechol reaction mixtures and authentic *cis,cis*-2-methylmuconic acid had characteristic infrared bands at 839 and 736 $cm.^{-1}$, bands different from those obtained with the cis,trans and trans,trans isomers. The UV spectra of the two biological products were also identical with those of the corresponding cis,cis acid, but they were different from those of the corresponding cis,trans and trans,trans acids.

Crystals of the product isolated after the enzymatic oxidation of 4-methylcatechol and authentic *cis,trans*-3-methylmuconic acid had identical infrared spectra and gas chromatographic retention times. The UV spectrum of the biological

Table I. Characteristics of Biologically Produced and Authentic Muconic Acids

Compound[a]	λ_{max}, mμ	$E \times 10^4$ at λ_{max}	Retention Time, Min.
Muconic acid			
Biological product	257	1.66	
Isolated product	257		5:50
Authentic (*cis,cis*)	257	1.61	5:50
Authentic (*cis,trans*)	260		6:07
Authentic (*trans,trans*)	260		7:27
2-Methylmuconic acid			
Biological product	262	1.73	
Isolated product	262		6:03
Authentic (*cis,cis*)	262		6:03
Authentic (*cis,trans*)	267	1.67	6:19
Authentic (*trans,trans*)	273		7:40
3-Methylmuconic acid			
Biological product	258	1.33	
Isolated product	265		7:01
Authentic (*cis,trans*)	265	1.68	7:01
Authentic (*trans,trans*)	264		8:56
3-Chloromuconic acid			
Biological product	261	1.41	
Isolated product	264		8:52
Authentic (*cis,trans*)	264	1.43	8:50
2,4-Dichloromuconic acid			
Biological product	269	1.48	
Isolated product	270		Unstable

[a] The biological product refers to the compound accumulating and analyzed in the incubation mixture. The isolated product refers to the compound obtained from ether extracts of the incubation mixture.

product was unlike that of authentic *cis,trans*- or *trans,trans*-3-methylmuconic acid and presumably corresponded to that of the cis,cis isomer (Table I).

A mixture containing two components was isolated after the enzymatic oxidation of 4-chlorocatechol. Extraction of this crude product with chloroform at room temperature, followed by removal of the solvent, gave a component which accounted for about one-sixth of the total. This compound was identified as the butenolide by comparison of retention times, UV, infrared, and mass spectra with those of an authentic sample (Table II). The insoluble residue, accounting for five-sixths of the total, showed an infrared spectrum and retention time identical to those of authentic *cis,trans*-3-chloromuconic acid. The UV spectrum of the biological product was unlike that of *cis,trans*-3-chloromuconic acid and presumably corresponds to that of the cis,cis isomer (Table I).

The biological product formed in the enzymatic degradation of 3,5-dichlorocatechol had a UV absorption maximum at 269 mμ, consistent with a muconic acid. The isolated product (λmax 270 mμ) was unstable and on repeated manipulation was totally converted to the chlorobutenolide, which was identified by comparison of its physical characteristics with those of an authentic sample (Table II). The infrared spectrum of the initial isolated product showed complex carboxyl absorption in the range 1680 to 1730 cm.$^{-1}$. The lower wavelength absorption (*ca.* 1720 cm.$^{-1}$) would be expected from a carboxyl group that had a chlorine atom attached to the α-carbon atom. A broad absorption band at *ca.* 910 cm.$^{-1}$, due to the acid dimer, was noted.

The mass spectrum of the isolated product was initially dominated by the more volatile chlorobutenolide. The chlorobutenolide spectrum decreased with time until it was almost totally replaced by that of the second component. A molecular ion with mass 210 was then observed. The 3 to 2 ratio of ion 210 to 212 indicated that the molecule contained two chlorine atoms. The presence of fragments with mass 193 and 165, containing two chlorine atoms, suggested loss of OH and CO plus OH, respectively. The existence of fragments with mass 175 and 157, containing one chlorine atom, suggested loss of Cl and Cl plus H_2O, respectively.

Formation of the butenolides from the chlorinated muconic acids was undoubtedly a nonenzymatic process, occurring after the incubation with the enzyme preparation had been terminated. As formation of the butenolides might also be catalyzed by the enzyme preparation, an attempt was made to separate the presumed delactonizing enzyme from the enzymes forming the butenolides by gel filtration on Sephadex G-200. However, all fractions capable of forming the muconic acids and butenolides contained an abundance of the delactonizing activity.

Metabolism of Muconic Acids. The metabolism of chlorinated muconic acids was measured by observing changes in the UV spectrum of the incubation mixture. When incubated with the enzyme for 3 hours, neither the isolated nor the authentic *cis,trans*-3-chloromuconic acid disappeared. On the other hand, a biologically accumulated sample of 3-chloromuconic acid, presumably the cis,cis isomer, was metabolized to yield a product with λ_{max} 242 mμ.

The isolated sample of 2,4-dichloromuconic acid, which was contaminated with <10% chlorobutenolide, was metabolized to yield a product with λ_{max} 260 mμ, the UV maximum being shifted to 272 mμ on acidification of the solution; this product was not investigated further. A biologically accumulated sample of 2,4-dichloromuconic acid, presumably the cis,cis

Table II. Evidence for the Identity of the Isolated Products as Chlorobutenolide and Butenolide

	Chlorobutenolide		Butenolide	
Analysis	Isolated product	Authentic compound	Isolated product	Authentic compound
Retention time (minutes)	4:37	4:38	3:20	3:20
Mass spectrometry				
Molecular ion, M	174	174	140	140
Fragments M−(OH)	157	157	123	123
M−(CO)	146	146	112	112
M−(Cl)	139	139	...	...
M−(CO + OH)	...	...	95	95
M−(2CO)	118	118	84	84
R_f-acidic[a]	0.59	0.60		0.61
R_f-basic[a]	0.89	0.89		0.89
Infrared bands (cm.$^{-1}$) above	1800	1800	1800	1800
1600 cm.$^{-1}$	1685	1685	1685	1685
	1635	1635	1645	1645

[a] R_f in thin-layer chromatograms in an acidic (benzene–ether–acetic acid, 6:3:1) and a basic (ethanol-water-NH_4OH, 100:25:3) solvent system.

Table III. Evidence for the Identity of Products Formed from Chlorocatechols and Butenolides as Maleylacetic Acids

Analysis	Chloromaleylacetic acid			Maleylacetic acid	
	Product of 3,5-dichlorocatechol metabolism	Product of chlorobutenolide metabolism	Synthetic compound	Product of 4-chlorocatechol metabolism	Product of butenolide metabolism
Retention time, minutes					
175° C.	17:20	17:27	17:32		15:05
200° C.				5:40[b]	5:38[b]
Mass spectrometry					
Fragments M−(CO_2)	148	148	148	114	114
M−(CO_2 + CH_3)	133	133	133	99	99
M−(2CO_2 + CH_3)	89	89	89		
UV spectrum					
λ_{max} (pH 7.2)	253	253	253	242	242
$E \times 10^3$ (pH 7.2)	9.60	9.47		4.44	4.20
R_f-acidic[a]	similar			0.44	0.45
R_f-basic[a]	0.74	0.75	0.76	0.77	0.77

[a] See Table II.
[b] Under identical conditions, retention time of authentic compound was 5:40 minutes.

isomer, was metabolized to yield a compound with λ_{max} 253 mμ, which was abolished on acidification.

Metabolism of the Butenolides. Both the butenolide and chlorobutenolide were rapidly acted upon by dilute extracts. For example, 0.5 μmole of the butenolide in 4.3 ml. of buffer was completely metabolized by 0.08 mg. of extract-protein within 10 minutes. Although rates of degradation were not compared, complete metabolism of 0.3 μmole of chlorobutenolide occurred within 1 minute in 6.0 ml. of buffer containing the same extract (0.06 mg. of protein). Chemical hydrolysis was shown not to be significant.

Accumulation and Identification of Maleylacetic Acids. When incubated with an appropriate concentration of the enzyme preparation, 4-chlorocatechol, *cis,cis*-3-chloromuconic acid and the butenolide gave a product which had the same UV spectrum (λ_{max} 242 mμ), a spectrum also identical with that of synthetic maleylacetic acid. In a similar manner, 3,5-dichlorocatechol, *cis,cis*-2,4-dichloromuconic acid, and the chlorobutenolide gave a product with λ_{max} 253 mμ, identical with that obtained for synthetic chloromaleylacetic acid. In all instances, UV absorption was essentially abolished on acidification of the incubation mixtures, resembling the keto-enol tautomerism noted by Lack (1959) with maleylpyruvic acid.

After isolation, the products formed in the metabolism of the chlorocatechols and the butenolides (λ_{max} 242 or 253 mμ) had identical chromatographic characteristics and infrared and mass spectra as synthetic maleylacetic or chloromaleylacetic acids (Table III). Molecular ions were not observed in the mass spectra of the maleylacetic acids, even at an ionization potential of 13 eV. The largest ion found represented an apparent loss of CO_2 from the proposed molecular ion. Fragmentation ions representing loss of CO_2, CO_2 plus CH_3, and 2 CO_2 plus CH_3 were also noted in both spectra.

Further evidence for the identity of maleylacetic acid was provided by an acid-catalyzed rearrangement of the synthetic compound to fumarylacetic acid. The physical characteristics (λ_{max} 228 and 292 mμ in ether and m.p. 158° C.) of the rearranged product corresponded well with published values for fumarylacetic acid (Elvidge *et al.*, 1950).

A second component was isolated in many experiments in which chloromaleylacetic acid had accumulated in the reaction mixtures. The retention time and infrared spectrum of this component were identical with those of a compound described by Bollag *et al.* (1968a) as a derivative of 2-chloro-4-methyl butyrolactone. The same compound was synthesized by alkaline hydrolysis of the chlorobutenolide followed by thermal decarboxylation of the chloromaleylacetic acid formed. The NMR spectrum of the synthetic compound showed *inter alia* a singlet for the methyl group resonance at τ 8.88 and a broad hydroxyl resonance at τ 5.57. The evidence presented by Bollag *et al.* (1968a), together with that presented here, indicates that the compound is the lactol form of *cis*-2-chloro-4-keto-pent-2-enoic acid.

Accumulation of β-Ketoadipic Acid. The extract (3.0 mg. of protein) was incubated with 2.0 μmoles of each of several possible substrates in 0.5 ml. of solution. Various tests were performed on the supernatant initially and after the 3-hour incubation period, following precipitation of the protein with 5% trichloroacetic acid. Analyses for catechol disappearance by the osmic acid procedure revealed that unsubstituted catechol, 4-chloro-, and 3,5-dichlorocatechol had totally disappeared, while 4,5-dichlorocatechol was not apparently transformed. Tests under alkaline conditions by the nitroprusside color reaction, which was positive for β-ketoadipic acid, indicated that such compounds were formed from catechol, *cis,cis*-muconic acid, muconolactone, 4-chlorocatechol, and 3,5-dichlorocatechol, although the color lasted only about 30 seconds with the compounds produced enzymatically from the last two substrates. Positive nitroprusside tests under acid conditions were given by β-ketoadipic acid itself and by the metabolites produced from catechol, *cis,cis*-muconic acid, and muconolactone, but not by the product of enzymatic attack on the two chlorocatechols. A 10-μl. aliquot from the reaction mixtures at the start and at the end of incubation of the enzyme with catechol, *cis,cis*-muconate and muconolactone was spotted on silica gel sheets, and the thin-layer chromatograms were developed in benzene–ether–acetic acid (10:3:1). Only one product was detected in each sample under UV light or by iodine absorption, and this compound had an R_f value identical to that of β-ketoadipic acid. Under the conditions employed, β-ketoadipic acid provided as a substrate for the enzyme was not metabolized.

Succinic Acid Formation. All of the original radioactivity introduced as 2,4-D remained in the aqueous phase at the end of the incubation period, and 60% of the substrate chlorine was released. One extraction with ethyl acetate removed 57% of the ^{14}C-containing material. Subsequent extractions removed 14 and 9%, respectively. Paper and thin layer chromatography (benzene–*p*-dioxan–acetic acid, 90:25:40) showed that the first extract contained unmetabolized 2,4-D and a small amount of product. The other extracts and the

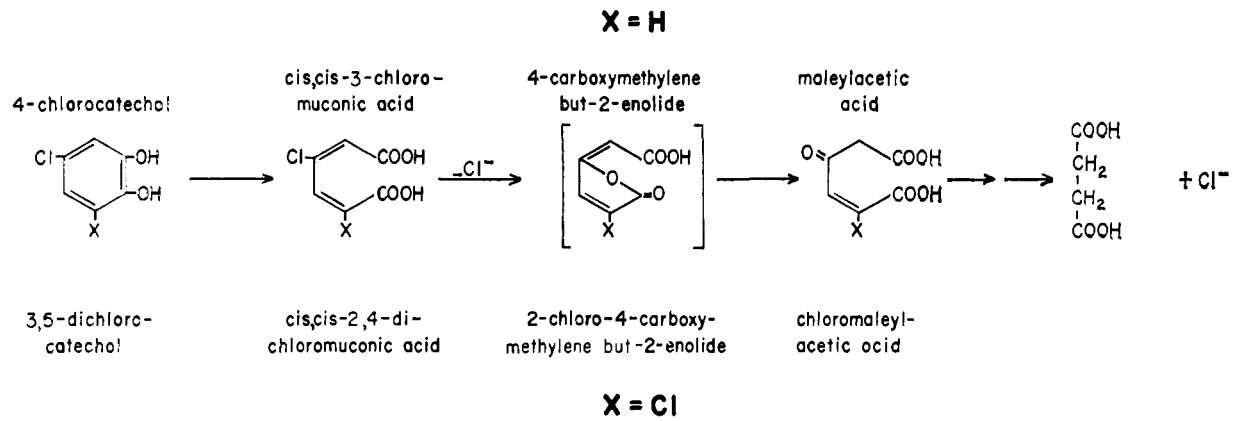

Figure 1. Pathway of chlorocatechol degradation by enzymes from *Arthrobacter* sp.

Hypothetical intermediates are shown in brackets

water phase contained no 2,4-D, but they did show one product. The R_f value of the single labeled product corresponded to the R_f of authentic succinic acid in the above solvent system and in *tert*-butanol–methyl ethyl ketone–formic acid–water (40:30:15:15), *tert*-butanol–methyl ethyl ketone–water–NH_4OH (4:3:2:1), water–saturated phenol and ethanol–water–NH_4OH (100:25:3). The product was separated in at least one solvent system from adipic, glutaric, pyruvic, lactic, oxalacetic, L-malic, and fumaric acids. In addition, the R_f values obtained for the product corresponded to those for succinic acid given by Fink *et al.* (1963), who characterized the chromatographic behavior of several hundred biological compounds in the solvent systems used.

DISCUSSION

Enzymes isolated from the *Arthrobacter* sp. grown on 2,4-D have been used to characterize the pathway of 2,4-D degradation. Previous investigations from this laboratory indicate that the ether linkage of 2,4-D is cleaved to yield 2,4-dichlorophenol and probably glyoxylic acid. The latter is converted to α-alanine, while the former is oxidized by a mixed function oxidase to 3,5-dichlorocatechol (Bollag *et al.*, 1968a; Loos *et al.*, 1967; Tiedje and Alexander, 1969). The results of the present study indicate that 3,5-dichlorocatechol, or the 4-chlorocatechol formed from 4-chlorophenoxyacetic acid, are dissimilated as shown in Figure 1.

Accumulation of the muconic acids was achieved by use of dilute extracts. The isolation of muconic acids as products indicates that the catechols are metabolized by an ortho-cleavage mechanism. With many muconic acids, the cis,cis isomer is unstable, and a nonbiological rearrangement to a more stable configuration might be expected to occur during the isolation procedure. Of the biologically formed cis,cis acids, only *cis,cis*-muconic acid and *cis,cis*-2-methylmuconic acid were stable enough to be isolated as such. Differences in the UV spectra of the biological and isolated products indicated that a change had indeed occurred during the isolation of 3-methyl- and 3-chloromuconic acids, which were obtained as the more stable cis,trans isomers. Moreover, the observation that the isolated *cis,trans*-3-chloromuconic was not a suitable substrate, whereas the biologically accumulated muconic acid was further metabolized, suggests, too, an isomerization to a nonutilizable form. A similar result has been reported by Evans and Moss (1957).

The configuration of the 2,4-dichloromuconic acid isolated after the enzymatic oxidation of 3,5-dichlorocatechol is not known, although it had a UV maximum very similar to that of the biological product. This dichloromuconic acid was acted upon by the enzyme preparation, but the final product (λ_{max} 260 mμ) was apparently different from that obtained from 3,5-dichlorocatechol under similar experimental conditions (λ_{max} 253 mμ). It therefore seems probable that the isolated 2,4-dichloromuconic acid did not have the cis,cis configuration.

A minor component was always isolated with the chlorinated muconic acids. This component apparently arose from the cis,cis acids by lactonization with displacement of chloride from the carbon atom beta to the carboxyl group, giving rise to 4-carboxymethylene but-2-enolide and 2-chloro-4-carboxymethylene but-2-enolide. The data do not suggest a biological accumulation of the butenolides, and indeed, such an accumulation is unlikely because the butenolides were rapidly hydrolyzed enzymatically, even when the amount of enzyme used for butenolide degradation was far less than that used in experiments in which the butenolides were isolated.

The butenolide and chlorobutenolide were isolated by Bollag *et al.* (1968a) as products formed during the enzymatic degradation of 4-chloro- and 3,5-dichlorocatechol, respectively. It now appears that the experimental conditions were such that the butenolides obtained in those studies were generated nonenzymatically from the corresponding muconic acids. Davies (1963) also isolated a compound formed during the enzymatic oxidation of 3,5-dichlorocatechol which was proposed to be the chlorobutenolide. The physical properties of Davies' compound are identical to those established in this study for the authentic chlorobutenolide. Davies separated the lactonizing enzyme from the delactonizing enzyme, and presented evidence that the chlorobutenolide is formed enzymatically, as well as chemically, from a compound he proposed was 2,4-dichloromuconic acid. The finding in the present study that butenolide and chlorobutenolide are converted enzymatically to malelyacetic acid and chloromaleylacetic acid, respectively, also supports the contention that the butenolides are involved in the metabolic pathway.

The maleylacetic acids were obtained enzymatically from the chlorocatechols, chlorinated muconic acids, and butenolides and by chemical hydrolysis of the butenolides. Although they were not isolated in a pure form, the analytical evidence strongly supports the proposed structures. Both maleylacetic and chloromaleylacetic acids had UV spectra very similar to published reports and infrared spectra showing strong carbonyl absorption. The mass spectrum of both compounds did not contain a molecular ion but instead a fragmentation ion representing loss of CO_2 from the expected molecular ion. Probably thermal decarboxylation of the β-

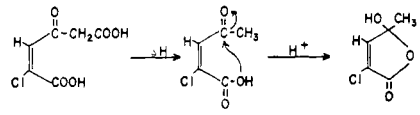

Figure 2. Formation of *cis*-2-chloro-4-keto-pent-2-enoic acid

keto acids occurred at the inlet temperature of the spectrometer. The fragmentation pattern of both compounds was consistent with the structure assigned.

In conditions favorable for chloromaleylacetic acid accumulation, another product, apparently not of biological origin, was often obtained following the extraction. The compound probably arose by thermal decarboxylation of the β-keto acid to give *cis*-2-chloro-4-keto-pent-2-enoic acid, which then formed the tautomeric cyclic lactol (chlorolactol) on acidification (Figure 2). The nonbiological origin of the chlorolactol was shown by its formation from synthetic chloromaleylacetic acid by this process, which is similar to the formation of levulinic acid from β-ketoadipic acid (Kilby, 1948). Levulinic acid may also exist in a lactol form (Finar, 1963). The chlorolactol was identical to a compound described by Bollag *et al.* (1968b). The isolation of the chlorolactol, which can only form if the carbonyl and carboxyl group involved have the cis arrangement, provides evidence that the parent compound had the cis configuration.

The identification of succinic acid as the final product of the reaction sequence catalyzed by the soluble enzyme preparation suggests that the terminal stages of the 2,4-D pathway involve the conversion of chloromaleylacetic acid to succinic acid. Evidence for such a conversion will be presented elsewhere.

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