2,4-D Metabolism: Enzymatic Conversion of Chloromaleylacetic Acid to Succinic Acid

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Enzymes obtained from a strain of *Arthrobacter* grown on 2,4-dichlorophenoxyacetic acid catalyzed the conversion of *cis*-1-chloro-3-keto-but-1-ene-1,4-dicarboxylic acid (chloromaleylacetic acid) and *cis*-3-keto-but-1-ene-1,4-dicarboxylic acid (maleylacetic acid) to succinic acid. The conversion was stimulated by the addition of substrate quantities of NADH or NADPH. Succinic acid was also formed enzymatically from other chlorinated compounds. A pathway for the conversion of maleylacetic acids to succinic acid in the last phase of phenoxyacetate herbicide detoxication is proposed.

The chemical 2,4-dichlorophenoxyacetic acid (2,4-D) is currently one of the most widely used herbicides in the world. Information on the fate of 2,4-D and related compounds in natural ecosystems is essential from both toxicological and ecological standpoints. Previous work from this laboratory has characterized the pathways for the conversion of 2,4-D to chloromaleylacetic acid (*cis*-1-chloro-3-keto-but-1-ene-1,4-dicarboxylic acid) and for the conversion of 4-chlorophenoxyacetic acid (4-CPA) to maleylacetic acid (*cis*-3-keto-but-1-ene-1,4-dicarboxylic acid) (Loos *et al.*, 1967; Bollag *et al.*, 1968a,b; Tiedje and Alexander, 1969; Tiedje *et al.*, 1969).

Little is known about the further enzymatic degradation of the maleylacetic acids, however. Although Fernley (1959) and Davies (1963) tentatively identified maleylacetic and chloromaleylacetic acids as products of 4-CPA and 2,4-D metabolism, respectively, they did not study the further metabolism of these intermediates. Fernley (1959) suggested that maleylacetic acid was isomerized to fumarylacetic acid, which was then cleaved to fumaric acid and acetyl-CoA, in a manner analogous to the metabolism of homogentisic and gentisic acids (Knox and Edwards, 1955; Lack, 1959). Davies (1963) suggested removal of the chlorine from chloromaleylacetic acid prior to its cleavage, as intact cells or extracts of the organism used would not attack chloromaleic, chlorofumaric, or chlorosuccinic acids. Moreover, Tiedje *et al.* (1969) reported the enzymatic formation of succinic acid from 2,4-D, suggesting that chloromaleylacetic acid is cleaved to a two- and a four-carbon fragment.

The present report is concerned with the enzymatic degradation of the maleylacetic acids formed as a result of the biological decomposition of phenoxy herbicides.

MATERIALS AND METHODS

The *Arthrobacter* sp. was grown in a 40-liter fermentor in the 0.2% 2,4-D-mineral salts medium described by Tiedje *et al.* (1969), and cell-free extracts were prepared as before. The enzymes catalyzing the metabolism of succinic acid were removed from the preparation by an initial centrifugation of the crude extract for 1.5 hours and a further 1-hour centrifugation of the resulting supernatant, both at 140,000 × G. The upper two thirds of the final supernatant was employed. The removal in this way of succinic dehydrogenase allowed succinic acid, when formed, to accumulate.

All substrates were dissolved in 0.02*M* potassium phosphate buffer, pH 7.3, and neutralized with NaOH. Reaction mixtures were incubated for 3 hours at 28° C, with shaking. Cofactor requirements were demonstrated using 2.5 μmoles of substrate and 10 mg of soluble extract with or without 2.5 μmoles of either NADH or NADPH in a 1.0-ml volume. Incubations were terminated by the addition of a pinch of Na_2WO_4 to aggregate the protein and 4*N* HCl to a pH of 1.5–2.0. The protein was removed by centrifugation, the supernatant continuously extracted with ether for 2 hours, and the products were isolated as previously described (Tiedje *et al.*, 1969).

To obtain sufficient quantities of succinic acid for isolation and chemical identification, 20 μmoles of 3,5-dichlorocatechol, 4-chlorocatechol, chlorobutenolide, and butenolide were incubated in a 10-ml volume separately with an amount of the enzyme preparation containing 200 mg of protein. Ten μmoles of 3-ketoadipic acid were incubated with 37 mg of extract-protein in a 10-ml volume. Succinic acid produced in the incubation mixtures was determined as the trimethylsilyl derivative by gas chromatography (Tiedje *et al.*, 1969). The isolated products were dissolved in 1–2 ml of ethanol and the solution diluted to 10 ml with ether. Five milliliters of the solutions were subsampled, and the solvent was removed using an N_2 purge. The residue was treated with 50 μl of *N,O*-bis-(trimethylsilyl)acetamide and diluted with 100 μl of CS_2. Four 2-μl injections were made into the chromatograph, and succinic acid was determined by comparing the average peak height of the succinic acid peak with a standard curve obtained by a similar procedure. Other acids were analyzed by similar procedures.

Thin-layer chromatography was performed on Eastman Chromagram silica gel plastic sheets or on plates made up with Merck silica gel, grade 7741. Acidic components were detected with a spray of 0.1% bromocresol green or 0.04% bromocresol purple in ethanol. Chloride was assayed by the method of Bergmann and Sanik (1957). Mass spectra were determined using an Associated Electrical Industries (Manchester, England) MS9 mass spectrometer equipped with a direct inlet system.

Chlorobutenolide, butenolide, maleylacetic acid, chloromaleylacetic acid, and fumarylacetic acid were synthesized as previously described (Tiedje *et al.*, 1969). Chlorofumaric acid (mp 183–185° C) and chloromaleic acid (mp 110–111° C) were synthesized by the method of Ashton and Partington (1934). 4-Chloro- and 3,5-dichlorocatechol were

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Table I. Effect of NADH on Enzymatic Formation of Succinic Acid from Various Substrates

Substrate	Cofactor	% Succinic Acid[a]
3-Ketoadipic acid	...	90
	NADH	50
Butenolide	...	12
	NADH[b]	74
Chlorobutenolide	...	10[c]
	NADH[b]	84
Fumarylacetic acid	...	19
	NADH[b]	26
Succinic acid	...	95
	NADH	60

[a] Percent recovery of substrate as succinic acid. [b] NADPH was equally effective. [c] In addition to succinic acid, *ca.* 50% of the substrate was recovered as *cis*-2-chloro-4-keto-pent-2-enoic acid (in the lactol form).

purified as previously described (Tiedje *et al.*, 1969), and 3-ketoadipic acid, NADH, and NADPH were obtained from Sigma Chemical Co.

RESULTS

The products isolated from reaction mixtures after incubation of the enzyme preparation with 3,5-dichlorocatechol, 4-chlorocatechol, chlorobutenolide, or butenolide all showed essentially a single component when analyzed by gas chromatography. The retention time of this component, 140 seconds, corresponded to the retention time of the trimethylsilyl derivatives of authentic succinic and maleic acids, namely 140 and 138 seconds. Organic acids that could be excluded were fumaric, chlorofumaric, chloromaleic, chlorosuccinic, malic, oxalacetic, and tartaric acids, which had retention times of 157, 156, 185, 190, 267, 340, and 490 seconds, respectively. Thin-layer chromatography (ethyl acetate–acetic acid–water, 3:1:1) indicated that the product from all four substrates was succinic (R_f *ca.* 0.8) rather than maleic (R_f *ca.* 0.6) acid. In these studies, the two butenolides were provided as substrates because, although they have not been shown conclusively to be intermediates in 2,4-D or 4-CPA metabolism, they are rapidly hydrolyzed enzymatically to the corresponding maleylacetic acids, less than 5 minutes being required for the hydrolysis. The butenolides are more easily synthesized and purified than the corresponding maleylacetic acids, moreover.

Mass spectrometric evidence confirmed the identification of succinic acid. Thus, low resolution mass spectra obtained on the product formed from chlorobutenolide and on succinic acid were identical. Major fragments with *m/e* 101 (P-OH), 100 (P-H_2O), 74 (P-CO_2), and 73 (P-COOH) were noted. The parent ion (*m/e* 118) was a minor component of the spectra.

To determine the cofactor requirements for the sequence involved in the enzymatic formation of succinic acid, the preparation was incubated with several substances in the absence or presence of NADH or NADPH. The quantities of succinic acid isolated at the end of the incubation period are shown in Table I. No succinic was recovered in enzyme preparations maintained for a like period without substrate. The results indicate that NADH or NADPH is a cofactor in the formation of succinic acid from maleylacetic and chloromaleylacetic acids. A six- to eight-fold increase in the production of succinic acid from the butenolides was observed when the pyridine nucleotides were used. The reduced yield of succinic acid when NADH is provided to an enzyme preparation supplemented with 3-ketoadipic acid is unexplained.

To determine the identity of possible intermediates generated during the microbial conversion of chloromaleylacetic acid to succinic acid, several substrates were incubated with the enzyme preparation alone and with NADH. The reaction mixture was examined quantitatively by gas chromatography for the presence of chlorosuccinic, chloromaleic, chlorofumaric, oxalacetic, maleic, fumaric, malic, and succinic acids. The results given in Table II show that succinic acid is formed most readily from chlorosuccinic acid, but small amounts are formed too from chloromaleic, chlorofumaric, oxalacetic, L-malic, and fumaric acid. The rate of conversion of the first two substrates was increased by NADH.

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 have established the metabolism of 2,4-D to chloromaleylacetic acid and of 4-CPA to maleylacetic acid (Tiedje *et al.*, 1969). The formation of ^{14}C-labeled succinic acid from ring-labeled 2,4-D was also demonstrated.

The pathway by which succinic acid is formed from the maleylacetic acids is suggested from the results of the present investigation. An enzymatic sequence analogous to the homogentisic acid and gentisic acid pathways of metabolism,

Table II. Metabolism of Possible Intermediates between Chloromaleylacetic Acid and Succinic Acid

Substrate	Cofactor	% Succinic Acid[a]	% Other Acids[a]	Total Recovery, %
DL-Chlorosuccinic acid	...	34	Chlorosuccinic (26%)	60
	NADH	46	Chlorosuccinic (30%)	76
Chloromaleic acid	...	14	...	14
	NADH	34	...	34
Chlorofumaric acid	...	11	...	11
	NADH	10	...	10
Oxalacetic	...	24	...	24
	NADH	14	...	14
L-Malic acid	...	15	Fumaric (5%)	20
	NADH	15	Fumaric (2%)	17
Fumaric acid	...	15	Fumaric (6%); Malic (12%)	33
	NADH	19	Fumaric (2%)	21
Succinic acid	...	95	...	95
	NADH	60	...	60
Endogenous	...	0	...	0

[a] Percent recovery of substrate as compound indicated.

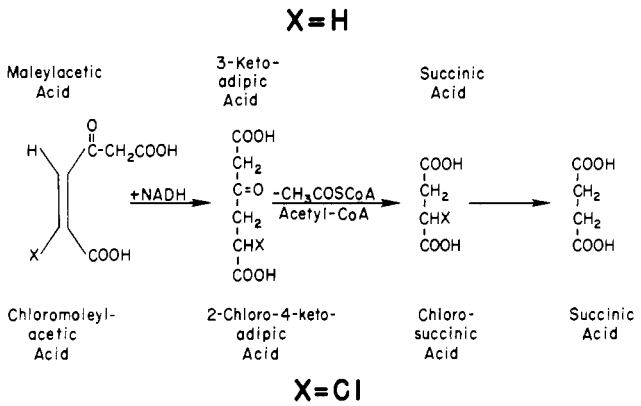

Figure 1. Proposed pathway for enzymatic degradation of maleylacetic acids

in which maleylacetoacetic acid (Knox and Edwards, 1955) and maleylpyruvic acid (Lack, 1959) are first isomerized to the fumaryl forms followed by cleavage to give fumarate as a common product, may be ruled out, as fumarylacetic acid was not readily transformed to succinic acid under the test conditions. More attractive is the view that the degradation involves an NADH- or NADPH-dependent reduction of the double bond in maleylacetic acid to give 3-ketoadipic acid. The latter would then be cleaved to yield acetyl-CoA and succinic acid (Figure 1). The observation that succinic acid was readily formed from 3-ketoadipic acid in the absence of added cofactor supports this proposal. It is also possible that the keto group of maleylacetic acid is reduced to give a β-hydroxy intermediate, which could then form muconolactone. The conversion of muconolactone to 3-ketoadipic acid by an enzyme preparation from this bacterium has been described (Tiedje *et al.*, 1969).

Any postulated pathway for the conversion of chloromaleylacetic acid to succinic acid must account for removal of the remaining chlorine. A likely possibility is the analog of the sequence proposed above, one involving an initial reduction of the double bond of chloromaleylacetic acid, giving rise to 2-chloro-4-ketoadipic acid. The finding that about half of the chlorobutenolide incubated with the enzyme preparation, in the absence of NADH, was recovered as the lactol form of *cis*-2-chloro-4-keto-pent-2-enoic acid, a chemical degradation product of chloromaleylacetic acid (Tiedje *et al.*, 1969), suggests that NADH is required for the further metabolism of chloromaleylacetic acid. The 2-chloro-4-ketoadipic acid generated may then be cleaved to give acetyl CoA and chlorosuccinic acid (Figure 1). The latter may then be dehalogenated, and indeed the enzyme preparation was found to convert DL-chlorosuccinic acid to succinic acid; not all of the DL-chlorosuccinate was dehalogenated, however, but the unmetabolized substrate possibly represented the enantiomer that was not readily attacked. The slow conversion of chloromaleic or chlorofumaric to succinic acid makes these compounds less attractive as intermediates.

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