Aerobic Cometabolism of DDT Analogues by *Hydrogenomonas* sp.

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A strain of *Hydrogenomonas* grown on diphenylmethane decomposed *p,p'*-dichlorodiphenylmethane as well as 1,1-diphenyl-2,2,2-trichloroethane. In the metabolism of diphenylmethane, *p,p'*-dichlorodiphenylmethane, and 1,1-diphenyl-2,2,2-trichloroethane, phenylacetic, *p*-chlorophenylacetic, and 2-phenyl-3,3,3-trichloropropionic acids were formed, respectively, but no chloride was released from the chlorine-containing substrates. Cleavage of the benzene ring of a DDT metabolite has thus been demonstrated. The ability of this bacterium to transform DDT and related compounds aerobically is governed by the presence of the *para*-chlorine and the substituent on the methylene-carbon.

The microbial metabolism of DDT [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane] has received little attention despite the widespread use of this insecticide and the likelihood that microorganisms are the chief agents of its degradation in nature. Barker and Morrison (1964) reported the conversion of DDT to DDD [1,1-bis-(*p*-chlorophenyl)-2,2-dichloroethane] in mouse tissue. Subsequently, Barker *et al.* (1965) isolated *Proteus vulgaris* from the intestinal flora and demonstrated that the conversion was bacterial and not the result of enzymes of the animal. Further studies have disclosed the importance of the microbial gut flora in metabolizing DDT fed to rats (Mendel and Walton, 1966).

Most studies involving DDT degradation have been concerned solely with its reductive dechlorination to DDD. However, Wedemeyer (1967a,b) demonstrated a series of enzymatic steps leading to the formation of *p,p'*-dichlorobenzophenone, a reaction sequence that involves the loss of but a single carbon atom with its three bound chlorines. The fate of the remaining 13 carbon atoms, the possibility of ring fission, and the identities of the products of further degradation remain unresolved, although Plapp *et al.* (1965) suggested that *p*-chlorobenzoic acid is generated from DDT. Nevertheless, the role of *p*-chlorobenzoic acid in DDT breakdown must be viewed as equivocal because of the lack of a rigorous identification of the compound.

The present report is concerned with the microbiologically effected ring fission of *p,p'*-dichlorodiphenylmethane, a product formed in DDT decomposition, and of two DDT analogues. In addition, the effect of chemical substituents upon ring cleavage of various diphenylmethanes is considered.

MATERIALS AND METHODS

Hydrogenomonas sp. was grown in a medium containing 2.0 g of diphenylmethane, 1.42 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 1.32 g of $(NH_4)_2SO_4$, 0.20 g of $MgSO_4$, 0.5 mg of $FeSO_4$ and 0.5 mg of $Ca(NO_3)_2$ per l. of water. The final pH was 7.4. To determine if the culture could use other substrates for growth, the mineral salts solution containing 0.05% of a carbon source was employed.

Cell suspensions were prepared from cultures grown at 30° C in 10 l. of media. Air was passed through the solution at a rate of 4 l. per min. Additional diphenylmethane (10 ml) was added aseptically after 16 hr. The bacteria were collected by centrifugation when the culture was 24 to 36 hr old, and the cells were washed three times in phosphate buffer, pH 7.2, and resuspended to an optical density that was constant in all experiments. Reaction mixtures containing 500 ml of cell suspension and 50 mg of substrate were incubated with shaking at 30° C for times ranging from 3 hr with diphenylmethane to 1 week with DDT. A control containing no substrate was included in each trial.

At the end of the incubation period, the cells were removed by centrifugation, and the supernatant was analyzed for catechols (Evans, 1947) and phenols (Harborne, 1964). The presence of chloride was determined by the addition of a few drops each of concentrated nitric acid and 0.1*M* $AgNO_3$ to 2 ml of sample. For identifying the reaction products, the supernatant was extracted with two 50-ml portions of hexane. The hexane was discarded, and the aqueous phase was acidified with 2.0 ml of 15*M* H_3PO_4 and then extracted with two 100-ml volumes of ether. The ether extracts were dried over anhydrous Na_2SO_4 and extracted three times with 50-ml volumes of 0.1*M* Na_2CO_3. The aqueous extracts were slowly acidified with H_3PO_4 and extracted twice with 30-ml volumes of ether. These ether extracts were dried over anhydrous Na_2SO_4 and taken to dryness.

Silica gel HF_{254} (Merck) prepared on 20 cm × 20 cm glass plates was used for thin-layer chromatography. The residues from the extractions were dissolved in a minimal amount of ether and spotted across the width of the silica gel plate. The plates were developed by ascending chromatography to a height of 16 cm in a solvent system of benzene, dioxane, and acetic acid (10:3:1, by volume). The separated bands were detected by means of an ultraviolet lamp at 254 nm. Each band was carefully scraped off the plate, and the compound was eluted from the silica gel with ether. This suspension was filtered through Whatman 42 filter paper and evaporated to dryness.

Melting point analyses were performed with a Nalge hot plate microscope. Infrared spectra were obtained with a double beam infrared spectrophotometer, Beckman model IR 10, using KBr disks containing 1 to 5 mg of the compound. Mass spectra were obtained with a Perkin Elmer 270 mass spectrometer equipped with a Honeywell 2106 visicorder using the solids probe inlet.

Benzhydrol, *p,p'*-dichlorodiphenylmethane, and phenylacetic acid were obtained from Eastman Organic Chemicals, Rochester, N.Y., and diphenylmethane, benzophenone, and all other halogenated compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis. Thin-layer chromatography showed that these chemicals were free of significant amounts of contaminating compounds, and hence they were used without further purification.

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RESULTS

Hydrogenomonas sp. grew on diphenylmethane, benzhydrol, and *p*-chlorobenzhydrol as sole carbon sources, although the ability to grow on the latter compound was feeble and was lost with time. DDT and *p,p'*-dichlorobenzophenone were neither utilized for growth nor cometabolized by cell suspensions. Washed cells did cometabolize *p,p'*-dichlorodiphenylmethane (DDM), *p,p'*-dichlorobenzhydrol, benzophenone, *p*-chlorobenzophenone, and 1,1-diphenyl-2,2,2-trichloroethane (DTE), however (Table I), with the accumulation of yellow-colored oxidation products that had absorption maxima of 395, 403, 423, 394, and 330 nm, respectively, in both 0.05*M* phosphate buffer, pH 7.2, and 0.01*M* NaOH. The colors were not evident in acidic solution (0.01*N* HCl). These characteristics are indicative of unsaturated keto-enol acids, which are formed upon ring cleavage of catechols at a point adjacent to one of the hydroxyls (Dagley *et al.*, 1960; Kojima *et al.*, 1961). Neither catechols nor phenols accumulated. Inasmuch as the bacterium possesses a constitutive metapyrocatechase and metabolized several substituted catechols, the failure to observe catechol accumulation is not unexpected.

Initial products of ring fission could not be purified by crystallization by the method of Kojima *et al.* (1961) or by thin-layer chromatography. Yellow oils, which did not crystallize and which darkened to deep reddish-brown colors after 24 hr, were frequently found. The oily products were tightly bound to the silica gel, and effective separation by even highly polar solvents could not be achieved.

The separation of certain phenylalkanoic acids was successful, however. The culture supernatant from cells grown on diphenylmethane was extracted and developed by thin-layer chromatography to give an intense band (R_f 0.68), which was eluted and analyzed. The R_f value, melting point (76–8° C), infrared spectrum, and mass spectrum of this substance were all identical to an authentic sample of phenylacetic acid. The same compound was also isolated from supernatants of cell suspensions incubated with diphenylmethane for 3 hr, and it was identified in the same manner.

A washed cell suspension incubated for 24 hr with DDM formed a metabolite which was separated and identified by the same techniques. The R_f value (0.64), melting point (102–4° C), infrared spectrum, and mass spectrum were identical to those of an authentic sample of *p*-chlorophenylacetic acid. The bacterium was able to use phenylacetic but not *p*-chlorophenylacetic acid as the sole carbon source for growth. Cell suspensions effected the cometabolism of *p*-chlorophenylacetic acid, as measured manometrically, and formed a yellow-colored product with an absorption maximum of 379 nm in neutral or basic solution. The absorption was abolished when the liquid was acidified. The bacteria released no chloride from this substrate.

A third metabolite appeared in cell suspensions incubated for 3 days with DTE. The properties of this compound were different from all reference chemicals examined. Its infrared spectrum (Figure 1) indicates that it is an aromatic acid. In the mass spectrometer, the metabolite decomposed as it reached the ionization chamber. The mass spectrum revealed a very large peak at *m/e* 36, representing HCl. The data from the mass spectrometer are given in Table II, using heights relative to that of the peak at *m/e* 102 as a basis for comparison. The ion at 216 contains two chlorine atoms, as indicated by the approximate 3:2:0.3 ratio of the $p:p+2:p+4$ peaks. Although only two chlorines were present in this substance, no chloride was released by the bacteria from the initial substrate.

Assuming the loss of HCl from the true parent ion, the molecular weight of the metabolite would be 252. The molecular weight and the fragmentation pattern presented in Table II indicating an aromatic ring, a carboxyl group, and three chlorines are consistent with the view that the metabolite is 2-phenyl-3,3,3-trichloropropionic acid. The lack of a C-Cl_3 band in the 710–780 cm^{-1} region of the infrared spectrum is not unusual (Bergmann and Pinchas, 1952).

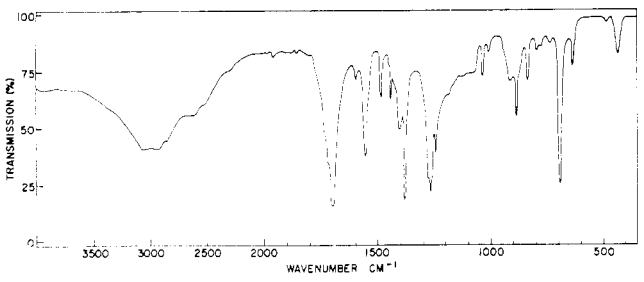

Figure 1. Infrared spectrum of degradation product formed from 1,1-diphenyl-2,2,2-trichloroethane

Table I. Growth and Aerobic Metabolism of DDT and Related Compounds by *Hydrogenomonas* sp.

Chemical Class	Test Compound	Growth	Aerobic Metabolism
Diphenylmethanes	Diphenylmethane	+	+
	p,p'-Dichlorodiphenylmethane (DDM)	−	+
Benzhydrols	Benzhydrol	+	+
	p-Chlorobenzhydrol	+	+
	p,p'-Dichlorobenzhydrol	−	+
Benzophenones	Benzophenone	−	+
	p-Chlorobenzophenone	−	+
	p,p'-Dichlorobenzophenone	−	−
1,1-Diphenyl-2,2,2-trichloroethanes	1,1-Diphenyl-2,2,2-trichloroethane (DTE)	−	+
	1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT)	−	−

Table II. Major Fragmentation Peaks in the Mass Spectrum of a Product Formed from 1,1-Diphenyl-2,2,2-trichloroethane

m/e	Relative Abundance	Apparent Explanation
216	13	—HCl
218	9	
220	1	
199	11	—HCl, OH
201	7	
203	1	
172	17	—HCl, CO_2
174	10	
176	2	
171	15	—HCl, COOH
173	10	
175	1	
145	77	—2HCl, Cl
136	80	—HCl, Cl, COOH
138	27	
135	24	—CCl_3
115	26	—O_2, Cl_3
102	100	—COOH, Cl_3
101	60	—HCl, COOH, Cl_2
89	28	—HCl, COOH, CCl_2
77	29	Remaining phenyl ion

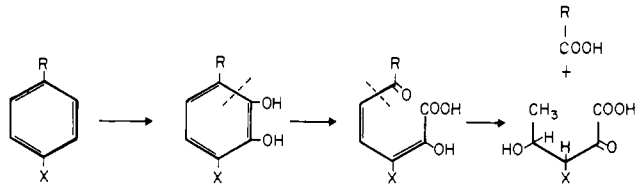

Figure 2. Hypothetical pathway for the metabolism of diphenylmethane (R = benzyl, X = H), DDM (R = *p*-chlorobenzyl, X = Cl), and DTE (R = 1-phenyl-2,2,2-trichloroethyl, X = H) by *Hydrogenomonas* sp.

DISCUSSION

The data indicate that presence of the *para*-chlorine and the substituent on the methylene-carbon govern the resistance of DDT to aerobic attack by *Hydrogenomonas* sp. The effect of aromatic chlorine substituents is evident in that: the bacterium was unable to grow upon DDM and *p,p'*-dichlorobenzhydrol, although it could utilize the corresponding monochloro and nonchlorinated compounds for growth; and it did not cometabolize DDT or *p,p'*-dichlorobenzophenone, whereas the monochloro and nonchlorinated analogues were cometabolized. The data show also that a trichloromethyl moiety or a carbonyl-carbon serves to make DDT and related compounds resistant to aerobic attack by this bacterium; thus, *Hydrogenomonas* sp. failed to metabolize DDT and dichlorobenzophenone, while it did act upon the corresponding compounds containing a methylene or CHOH bridge between the two benzene rings. The effect of chemical structure on the suitability of these compounds for microbial attack has an interesting chemical parallel. For example, aromatic chlorine substituents strongly withdraw electrons from the ring and make it less reactive, while an oxygen or hydroxyl on the bridge carbon has a slight deactivating effect (Morrison and Boyd, 1969). Methyl and benzyl groups, on the other hand, contribute electrons to the ring and make it more reactive.

In light of the finding that diphenylmethane, DDM, and DTE are converted biologically to phenylacetic acids, one of the benzene rings in each of the substrates unquestionably is cleaved. A likely initial step in this metabolic sequence is an oxidation to yield a benzyl catechol, which might then be decomposed in much the same way that toluene is metabolized through 3-methylcatechol (Figure 2), as reported by Dagley *et al.* (1964) and Gibson *et al.* (1968). The hydroxyls of the presumed catechol generated from DDM, a product formed biologically from DDT (Wedemeyer, 1967a,b), probably are not introduced onto carbons 3 and 4 because the chlorine substituents of DDM would block the formation of such a catechol. Moreover, cleavage of the hypothetical catechol produced in DDM degradation could not occur between the chlorine and adjacent hydroxyl-bearing carbons because an acyl chloride would be formed, and this would decompose spontaneously in water to liberate chloride, an anion not produced by this bacterium under the test conditions.

The observation that *Hydrogenomonas* sp. grows on diphenylmethane or phenylacetic acid but not on DDM or *p*-chlorophenylacetic acid, although it is capable of metabolizing them, suggests that the complete degradation of DDM to CO_2 and H_2O cannot be accomplished because of the organisms' inability to dehalogenate one or more chlorine-containing products formed from ring cleavage. Such products might accumulate in nature, and hence an investigation of their identities would be of considerable importance to an understanding of the fate of DDT in natural ecosystems.

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