Dechlorination of (2,4,5-Trichlorophenoxy)acetic Acid by Anaerobic Microorganisms

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An anaerobic methanogenic consortium that was grown in a mineral salt medium on 3-chlorobenzoate metabolized (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T). HPLC, MS, and NMR analysis showed that 2,4,5-T was dechlorinated at the para position to form (2,5-dichlorophenoxy)acetic acid (2,5-D). 2,5-D was not metabolized by the enrichment microorganisms nor were several other chlorinated phenoxyacetic acids. No transformation of 2,4,5-T was catalyzed by autoclaved cells. The rate of 2,4,5-T dechlorination was unaffected by the presence of equimolar concentrations of (2,4-dichlorophenoxy)acetic acid, dicamba, or a combination of the two chemicals. However, the addition of autoclaved or fresh sludge to the bacterial enrichment stimulated the rate of 2,4,5-T metabolism. These findings show that this 3-chlorobenzoate-grown consortium can dechlorinate a compound other than a halogenated benzoate and that it removed the para chlorine unstead of the meta chlorine, which characterized the consortium's activity on chlorobenzoates. Anaerobes may possess capacities to degrade some xenobiotic compounds that are considered recalcitrant under aerobic conditions.

The phenoxy herbicides are highly selective pesticides that are often used in combination with each other, or with other xenobiotic compounds like dicamba (3,5-dichloro-*o*-anisic acid), for the control of undesirable plant species. These widely used compounds have come under public scrutiny owing to their potential toxicological hazard (Grant, 1979; Hanify et al., 1981) and the resistance of some chemicals in this class to aerobic microbial degradation.

The environmental fate of (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T) has recently attracted particular attention since, in contrast to some of the other phenoxy herbicides, there are no known microorganisms in the natural environment that are capable of using 2,4,5-T as a sole carbon and energy source. It is generally believed that 2,4,5-T can only be slowly degraded by microorganisms through co-oxidative metabolism (Horvath, 1970; Rosenberg and Alexander, 1980a,b). This recalcitrance prompted Charkrabarty and co-workers to use "plasmid assisted molecular breeding" to enrich a mixed culture of 2,4,5-T-degrading microorganisms (Kellogg et al., 1981) and eventually to isolate a psuedomonad capable of growth on 2,4,5-T (Kilbane et al., 1982).

Previous reports of 2,4,5-T degradation have employed only aerobic incubations. Consequently, only the oxidative pathways for its metabolism have been studied. There is a paucity of information on potential reductive transformations of 2,4,5-T. Studies which purport to show 2,4,5-T metabolism in reducing environments often use ill-designed anaerobic conditions and report the oxidative conversion of 2,4,5-T to the corresponding trichlorophenol (Eder, 1980).

We recently reported on the metabolic versatility of an enriched anaerobic microbial consortium isolated from sewage sludge by their ability to completely mineralize 3-chlorobenzoic acid (Suflita et al., 1982). We found that the consortium could degrade a number of halogenated benzoates through successive aryl dehalogenations. We now extend our observations to another class of halogenated aromatic compounds. In this article, we report the first evidence for the reductive metabolism of 2,4,5-T in vitro and in anoxic sewage sludge slurries by a consortium of methanogenic microorganisms.

MATERIALS AND METHODS

Microorganisms and Incubation of Substrates. An anaerobic consortium of microorganisms consisting of both chemolithotrophic and heterotrophic methanogens and several unidentified Gram-negative rods was used in this study (Suflita et al., 1982). The consortium was originally enriched from sewage sludge through its ability to mineralize 3-chlorobenzoic acid to CH_4 and CO_2. The cells were cultured by using the mineral salt medium of Zehnder and Wuhrmann (1977). Resazurin was used as a redox indicator. The initial gas phase was an O_2-free mixture of 90% N_2–10% CO_2.

The bacterial cells were grown on 3-chlorobenzoate to a density of 30–50 μg of protein mL^{-1}, as measured by the method of Lowry et al. (1951), before 100 mL of culture was transferred to sterile 160-mL serum bottles by using the modified Hungate technique (Bryant, 1972). No 3-chlorobenzoate was present in the bottles at the start of the experiments as verified by high-pressure liquid chromatography (HPLC). The substrates were dissolved in dilute alkali and injected by syringe into the stoppered serum bottles to achieve a final concentration of 200–350 μM. All incubations were performed at 37 °C in the dark with constant mixing (110 rpm). The 2,4,5-T metabolite was isolated from 600 mL of culture grown in a 1-L incubation vessel (Suflita et al., 1983). Autoclaved cultures, amended with the various test substrates, remained reduced throughout the experiments and served as sterile anaerobic controls. All experiments were performed in duplicate.

Sludge Amendments. Primary anaerobic sludges from the Holt and Jackson, Michigan, municipal waste treatment facilities were collected and stored as previously described (Horowitz et al., 1982). Sludge was filtered through a layer of cheesecloth and 10 mL was added to serum bottles inside an anaerobic glovebox; the bottles were stoppered and subsequently removed from the glovebox. To test the ability of the consortium to degrade 2,4,5-T in sludge, we amended autoclaved (twice for 1 h) sludge and fresh sludge with 0, 45, or 90 mL of cells from the 3-chlorobenzoate-degrading consortium using strict anaerobic techniques (Bryant, 1972). Sterile reduced mineral salt medium was then used to adjust the final volume in the serum bottles to 100 mL. Incubation conditions, substrate concentrations, and initial headspace

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atmosphere were as described above. The rate of 2,4,5-T degradation by the consortium in sludge-amended bottles was compared with the rate found for the consortium diluted by the same amount with the mineral salt medium.

Chromatography and Isolation of Metabolite. Substrate disappearance and product appearance were followed by periodically sampling 23 mL from the serum bottles, passing the sample through a 0.22-μm membrane filter (Millipore, Bedford, MA), and storing the filtrate at −10 °C before HPLC analysis. Reversed-phase separations were performed on a Varian 5000 liquid chromatograph as previously described (Suflita et al., 1983), but a Zorbax C-8 column (25 cm by 4.6 mm i.d.; Du Pont Instruments, Wilmington, DE) was also used with a 1:1 methanol–50 mM acetate buffer (pH 4.5) mobile phase. For some compounds, slight modifications in the ratio of mobile phase components was necessary to achieve efficient peak separation. A variable-wavelength UV spectrophotometer was used to detect the compounds (Suflita et al., 1983).

The culture was incubated for about 1 week to isolate the 2,4,5-T metabolite. By that time the parent substrate was completely degraded as determined by HPLC. The culture medium was then acidified to pH 2.0 with dilute HCl and extracted twice with equal volumes of diethyl ether. Decolorizing carbon was added to the ether phase and the ether extract was dried over anhydrous Na_2SO_4. After removal of the carbon by filtration, the ether was evaporated to dryness and the residue was taken up with methanol, decolorized again, and evaporated to dryness.

Identification of Product. The mass spectrum of the 2,4,5-T product was obtained by direct probe on a Finnigan Model 4023 mass spectrometer equipped with an INCOS data system and operated at an ionizing potential of 70 eV. The proton nuclear magnetic resonance (NMR) spectrum of the isolated metabolite was taken on a Varian EM-360 MHz instrument using a sweep width of 10 ppm. The sample was dissolved in Me_2SO-d_6. The NMR spectrum and HPLC mobility of the metabolite were compared with those of the authentic compound synthesized according to the method of Vogel (1956) and purified from unreacted starting material by HPLC. The appropriate HPLC fractions were pooled, acidified, and extracted with ether as previously described to isolate the authentic material. A Varian T-60 NMR spectrometer was used to record the spectrum of the chemically synthesized material dissolved in Me_2SO-d_6.

Chemicals. 2,4,5-T and (2,4-dichlorophenoxy)acetic acid (2,4-D) were obtained from Sigma Chemical Co., St. Louis, MO. (3,4-Dichlorophenoxy)- and (2,3-dichlorophenoxy)acetic acid (3,4-D; 2,3-D), (4-chlorophenoxy)acetic acid, phenoxyacetic acid, 2,5-dichlorophenol, 2,4-dichlorophenol, and 2,4,5-trichlorophenol were purchased from Aldrich Chemical Co., Milwaukee, WI. Dicamba was a gift from the Velsicol Chemical Co., Chicago, IL.

RESULTS

HPLC analysis of the filtered culture medium revealed that 2,4,5-T was degraded when supplied to the consortium as the sole carbon substrate (Figure 1). Autoclaved cells also kept under similar reducing conditions did not alter the substrate. When 2,4-D (Figure 1), dicamba, or 3-chlorobenzoate were added together with 2,4,5-T, no effect on the 2,4,5-T degradation rate was noted. The initial degradation rate was nearly constant, indicating zero-order kinetics in this region; this rate was 0.102 μM h^{-1} (mg of $protein^{-1}$). Concurrent with the disappearance of 2,4,5-T was the appearance of a second HPLC peak with a shorter retention time (Figure 1; 2.26 min.). This peak was not detected in the autoclaved control. The metabolite peak

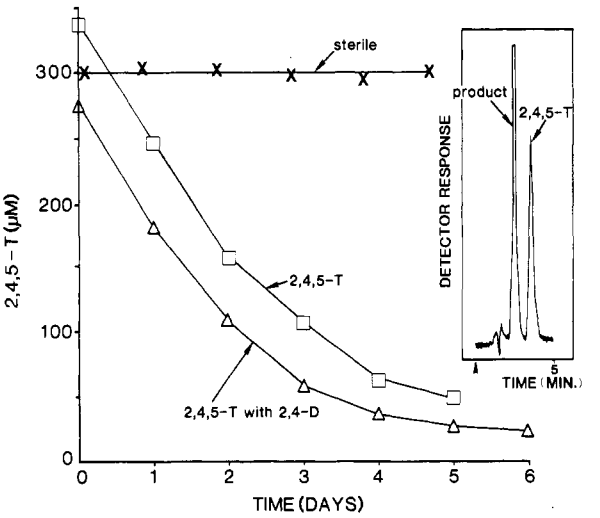

Figure 1. Disappearance of 2,4,5-T in a mineral salt medium when incubated with the anaerobic consortium in the presence and absence of 2,4-D. The absence of any degradation by the autoclaved consortium maintained at low redox conditions is also shown. The HPLC chromatogram (inset) shows the appearance of a product of shorter retention time than the 2,4,5-T substrate.

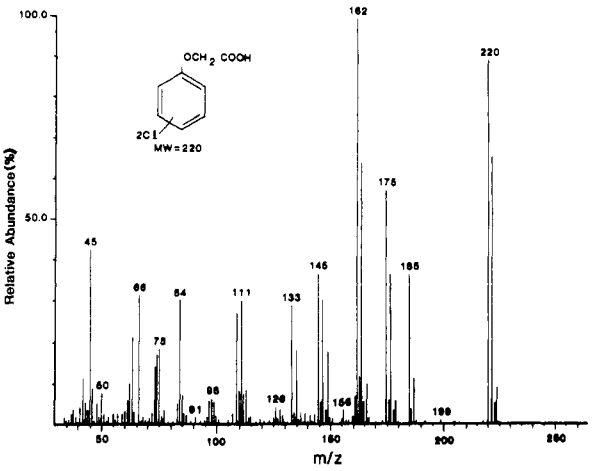

Figure 2. Mass spectrum of the isolated metabolite formed when 2,4,5-T was incubated with the anaerobic consortium.

increased until no 2,4,5-T was detected in the culture medium and it did not diminish even after extended incubation (20 days).

The metabolite was extracted from the culture filtrate and its mass spectrum determined. As can be seen in Figure 2, the metabolite shows several mass spectral features with a two chlorine isotopic abundance pattern (m/z 162, 175, 220), no three chlorine patterns, a base peak at m/z 162, and a parent ion at m/z 220. Since the metabolite was formed from a parent molecule with three chlorine atoms, the simplest structure consistent with the mass spectral data is the substitution of an aryl chlorine with a proton to form a (dichlorophenoxy)acetic acid (Figure 2).

The (dichlorophenoxy)acetate was further defined by its chromatographic mobility and NMR spectrum. The metabolite peak did not cochromatograph with either 2,4-D or 3,4-D, suggesting that the meta and ortho chlorines, respectively, were not removed. However, the metabolite did cochromatograph with 2,3-D even though the latter cannot be produced from 2,4,5-T barring some unanticipated intramolecular rearrangement of the haolgens. The loss of the para chlorine would result in (2,5-dichlorophenoxy)acetic acid (2,5-D), and like 2,3-D it also contains

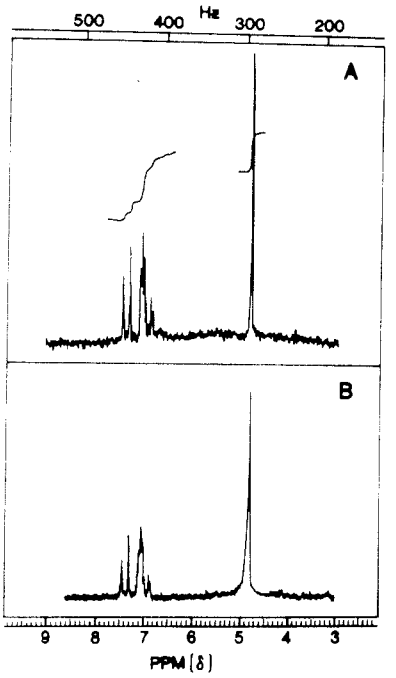

Figure 3. Comparison of NMR spectra for the isolated 2,4,5-T metabolite (A) and authentic 2,5-D (B).

an ortho and a meta chlorine and should exhibit similar chromatographic behavior.

Therefore, authentic 2,5-D was synthesized by using chloroacetic acid to acetylate 2,5-dichlorophenol. The NMR spectrum of the metabolite and its HPLC retention time were compared with those of authentic 2,5-D. The metabolite cochromatographed and exhibited NMR features identical with those of authentic 2,5-D (Figure 3). Hence, 2,4,5-T was dechlorinated by the anaerobic consortium in the para position to form 2,5-D.

Similar experiments and HPLC analysis revealed that the consortium was unable to transform 2,4-D, 2,3-D, 2,5-D, (4-chlorophenoxy)acetic acid, phenoxyacetic acid, 2,4-dichlorophenol, 2,4,5-trichlorophenol, or dicamba.

No degradation of 2,4,5-T was detected during a 5–6-week incubation in a 10% slurry of either Holt or Jackson anaerobic sludge (Figure 4). However, 2,4,5-T was transformed (and 2,5-D produced) when various amounts of the dehalogenating enrichment culture were incubated with the sludge. The extent of degradation was proportional to the size of the enrichment inoculum (Figure 4). The presence of the sludge in the enrichment culture stimulated the rate of 2,4,5-T dehalogenation over that found for the same concentrations of enrichment in mineral salt medium. Autoclaved sludge stimulated the degradation to about the same extent as did fresh sludge (Figure 4).

DISCUSSION

These data demonstrate the potential for reductive metabolism of 2,4,5-T by anaerobic microorganisms. The mass spectrum (Figure 2) confirms that 2,4,5-T was dehalogenated by the anaerobic consortium in a similar manner to that previously described for a variety of halogenated benzoic acid molecules (Suflita et al., 1982). Dehalogenation reactions by this chlorobenzoate grown consortium are therefore not limited to halobenzoate substrates. However, unlike halobenzoates, which preferentially lose meta-substituted halogens, the consortium dehalogenated 2,4,5-T at the para position as indicated by the NMR spectrum (Figure 3). The product (2,5-D), also with a meta halogen, was not further metabolized by the enriched bacteria.

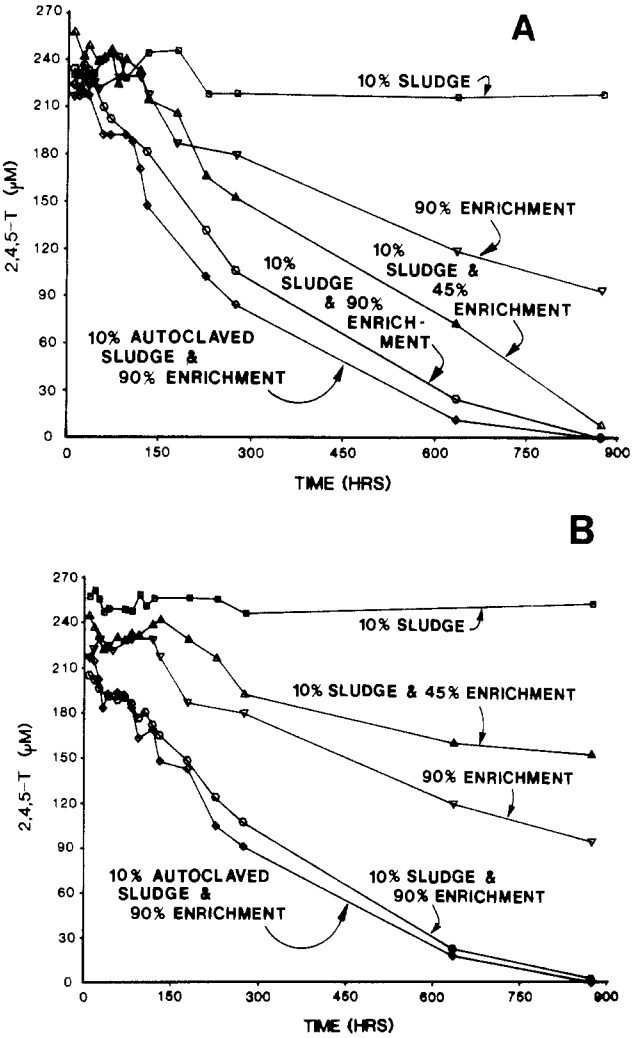

Figure 4. Rate of 2,4,5-T metabolism by the dehalogenating consortium mixed in 45% and 90% portions with 10% Jackson (A) and Holt (B) sludges or with mineral salt medium. Coefficients of variations were generally less than 5%.

Since the microorganisms could not mineralize the substrate carbon, it is unlikely that energy was derived from 2,4,5-T metabolism. Although the product was not further metabolized by this consortium, it is likely that it would be degraded aerobically by a pathway similar to that found for 2,4-D metabolism (Tiedje and Alexander, 1969). Thus, a scheme of sequential anaerobic and then aerobic treatment should result in complete mineralization of 2,4,5-T.

The rate of 2,4,5-T dehalogenation exhibited by the anaerobic consortium is about one-third of that previously reported for the growth substrate, 3-chlorobenzoic acid (Suflita et al., 1982). The rate of anaerobic 2,4,5-T degradation was 0.1 μM h^{-1} (mg of $protein^{-1}$), which is approximately 1 order of magnitude less than the estimated rate for the aerobic organism obtained by plasmid assisted molecular breeding (Kilbane et al., 1982). However, the anaerobic rate is for the total consortia and would be expected to be higher if it could be expressed for only the organisms active in dechlorination. Furthermore, no special genetic techniques were required to obtain 2,4,5-T metabolism by the anaerobic bacteria in this study.

Since 2,4,5-T is often used in combination with 2,4,-D and/or dicamba, it is important to understand whether the latter two herbicides affect the decomposition of the former. We expected this might occur since we previously observed that 3,5-dichlorobenzoate competitively inhibited the dehalogenation of 3-chlorobenzoate (Suflita et al.,

1983). Our results indicate that neither 2,4-D, dicamba, nor a combination of the chemicals significantly affected 2,4,5-T metabolism by the dehalogenating consortium. To be useful the consortium must be able to dehalogenate 2,4,5-T in more complex anaerobic environments where a host of biotic and abiotic factors can interact and potentially influence biodegradation. To provide a preliminary indication of this ability, we incubated the consortium in autoclaved and fresh sludge diluted to 10%. Our results indicate that not only can 2,4,5-T still be dehalogenated under these conditions but also the activity was stimulated compared to that of sludge-free controls (Figure 4). Since there was no significant difference in 2,4,5-T degradation between autoclaved and fresh sludge amended cultures, it is likely that the heat-stable component(s) of sludge is (are) responsible for the observed stimulation in dehalogenation. A similar stimulation has been observed with autoclaved and fresh anoxic sediment amended with the consortium (unpublished observations).

The anaerobic bacteria present in the dehalogenating consortium have been cultured in vitro for almost 2 years. The only previous exposure of these cells to 2,4,5-T may have occurred in the sewage sludge from which they were originally enriched. However, even after many generations without exposure to 2,4,5-T, the cells still possess the ability to transform this substrate. Anaerobic microorganisms may possess degradative enzymes for metabolizing xenobiotic molecules that are considered to be recalcitrant under aerobic conditions. The biodegradation potential of anaerobes, and the factors affecting their metabolic transformations, needs to be more extensively investigated.

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Registry No. 2,4,5-T, 93-76-5; 2,5-D, 582-54-7; 2,4-D, 94-75-7; dicamba, 1918-00-9.

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Herbicidal and Plant Growth Regulant Active 2-Sulfonylpyridine 1-Oxides

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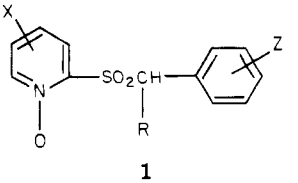
A series of 2-benzylsulfonylpyridine 1-oxides is described in which the identity of substituents on the pyridine ring, on the benzene ring, and in the α-benzyl position is varied. Preparation via several routes is described including the following: (a) condensation of a 2-halopyridine or a 2-halopyridine 1-oxide with a benzyl mercaptan followed by oxidation; (b) condensation of a 2-mercaptopyridine with a benzyl halide followed by oxidation; (c) a novel three-step one-pot reaction involving metalation, sulfurization, and alkylation of a pyridine 1-oxide unsubstituted in the 2-position. A clear relationship is shown to exist between herbicidal activity and the identity of substituents on each part of the molecule. Good turf growth retardation activity is also described for some compounds in this series.

The noval class of biologically active compounds, the 2-sulfonylpyridine 1-oxides, was recently reported (Plant and Bell, 1976). A wide variety of substituents was reported on the sulfonyl group, but no additional substitutents were attached to the pyridine ring. We have explored the area of chemistry described by structure 1, in which



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substituents on both the pyridine ring and the benzene ring were varied. This series was shown to possess good herbicidal activity, primarily against grasses, with good crop selectivity and up to 3 months residual. In addition, a

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