Cometabolism of the Herbicide 2,3,6-Trichlorobenzoate

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An organism, identified as a *Brevibacterium* sp., was isolated and was shown to degrade the herbicide 2,3,6-trichlorobenzoate by a cometabolic process. The oxidative pathway appeared to proceed through 2,3,6-trichloro-4-hydroxybenzoate and 2,3,5-trichlorophenol to the end product 3,5-dichlorocatechol, which accumulated in the media. 3,5-Dichlorocatechol was toxic to whole cells but did not inhibit the 2,3,6-trichlorobenzoate oxidizing enzymes or the pyrocatechase enzyme. Specificity of the pyrocatechase enzyme for an unsubstituted catechol was the cause of the phenomenon of cometabolism exhibited by this isolate.

The herbicide, 2,3,6-trichlorobenzoate (2,3,6-TBA) is a potent growth regulator producing drastic formative effects, similar to but not identical with those of 2,4-dichlorophenoxyacetate (Crafts, 1957). Studies on the microbial decomposition of 2,3,6-TBA had shown that, in comparison with 2,4-D, it was very resistant to bacterial degradation (Sheets *et al.*, 1968). MacRae and Alexander (1965) showed that 2,3,6-TBA was not significantly degraded by soil microorganisms in 60 days, whereas this same soil suspension completely metabolized benzoate in 3 days.

The recalcitrant nature of this herbicide results from both the number of chlorines attached to the aromatic nucleus and the position of these chlorine substituents on the benzene ring. MacRae and Alexander (1965) reported rapid degradation of all monochlorobenzoates by a soil suspension, although this soil suspension did not attack 2,4-, 3,4-, and 2,5-dichlorobenzoates 2,3,4-, 2,3,5-, 2,3,6-, and 2,4,5-trichlorobenzoates, or 2,3,4,5-tetrachlorobenzoate. In addition to the effect of the number of chlorines attached to the ring, a chlorine in the 3 or 5 position appears to impart resistance to bacterial degradation to the molecule (Alexander, 1964; Alexander and Aleem, 1961; Burger *et al.*, 1962).

Although 2,3,6-TBA was thought to be refractory to microbial degradation, it appeared that this compound was subject to slow inactivation in many soils (Sheets *et al.*, 1968). Dewey *et al.* (1962) showed that 2,3,6-TBA was degraded by biologically active soil with the release of 30 to 50% of the chlorine as inorganic chloride. No intermediate compounds or end products were detected or identified.

Isolation of an organism capable of using 2,3,6-TBA as a sole source of carbon and energy for growth had not been reported. This indicated that degradation of 2,3,6-TBA in nature might be accomplished by a cometabolic reaction. Evidence is available indicating that cometabolism may be an important phenomenon in the breakdown of pesticides (Alexander, 1967). The widespread occurrence of the phenomenon in nature had been suggested (Horvath and Alexander, 1970) and would support the idea of its importance in pesticide degradation.

This investigation attempted to demonstrate the cometabolic degradation of 2,3,6-trichlorobenzoate, the mechanism involved in cometabolism of this herbicide, and the pathway of its degradation.

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MATERIALS AND METHODS

The organism employed in this study was isolated by enrichment culture technique as previously described (Horvath and Alexander, 1970). The isolate was characterized according to "A Guide to the Identification of the Genera of Bacteria" (Skerman, 1967). This *Brevibacterium* sp. was cultured on the benzoate-salts agar described earlier (Horvath and Alexander, 1970).

2,3,6-Trichlorobenzoate was quantitated by the spectrophotometric method of Alexander and Lustigman (1966). 3,5-Dichlorocatechol was measured by the method of Arnow (1937). Inorganic chloride was determined according to the procedure of Bergmann and Sanik (1957).

Standard manometric techniques (Umbreit *et al.*, 1964) were employed to measure O_2 uptake and CO_2 release. Results are corrected for endogenous respiration unless otherwise noted.

Thin-layer chromatography employed Eastman Chromagram silica gel coated plastic sheets with fluorescent indicator. Butanol-benzene-water (1:9:10), butanol-acetic acid-water (4:1:5), and chloroform served as the solvent systems. All chromatograms were developed to a height of 50 mm. Spots were detected under ultraviolet light.

Lead derivatives of the sample and authentic 3,5-dichlorocatechol were prepared according to the method of Helling and Bollag (1968). Infrared spectra of these derivatives were determined as KBr macropellets in a Beckman Model IR 10 spectrophotometer.

Cell free systems were prepared as previously described (Horvath and Alexander, 1970). Respirometry was used to determine activity of these preparations on the compounds under study. Flasks containing boiled enzyme preparation or no enzyme preparation were included as controls.

2,3,6-Trichlorobenzoate and 3,5-dichlorocatechol were obtained from K & K Laboratories, Inc., Plainview, N.Y. 2,3,6-, 2,4,5-, and 3,4,5-trichlorophenols were supplied by Amchem Products, Inc., Ambler, Pa. The J. T. Baker Chemical Co., Phillipsburg, N.J., was the source of benzoate and acetic acid. Analytical reagent grade chloroform, butanol, and benzene were manufactured by Mallinckrodt Chemical Works, New York, N.Y.

RESULTS AND DISCUSSION

One of the 20 isolates previously described was shown to oxidize 2,3,6-trichlorobenzoate with the uptake of 1 μmole of oxygen per μmol of 2,3,6-TBA (Horvath and Alexander, 1970), although the organism was not able to use this herbicide as a sole source of carbon and energy for growth in a mineral-salts medium. The oxidation of 2,3,6-TBA by the isolate occurred, therefore, by a cometabolic mechanism.

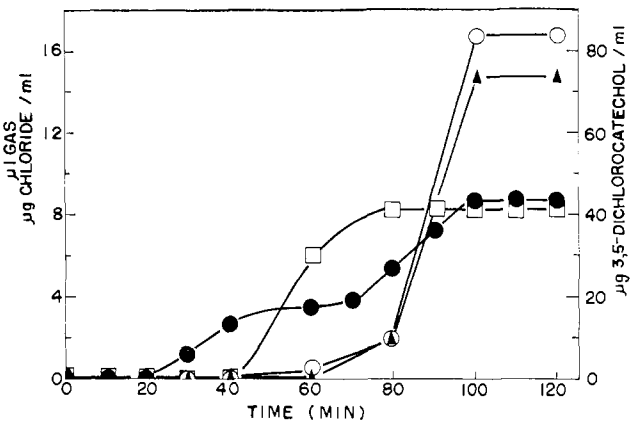

Figure 1. Oxygen uptake (●), CO_2 release (□), inorganic chloride release (○), and 3,5-dichlorocatechol production (▲) by a resting cell suspension of a *Brevibacterium* sp. cometabolizing 0.1 mg 2,3,6-trichlorobenzoate

The organism, identified as a *Brevibacterium* sp., was a nonsporing, gram positive rod which was motile *via* peritrichous flagella. It produced acid but no gas from glucose, liquefied gelatin, reduced and peptonized litmus milk, but did not produce indole, and exhibited a white effuse growth on nutrient agar slants.

Oxidation of 2,3,6-TBA occurred in a bimodal fashion with the first rise in oxygen uptake corresponding to 0.5 μmole per μmole of 2,3,6-TBA, and the second rise equalling an additional 0.5 μmole of oxygen per μmole of herbicide (Figure 1). An initial adaptive lag period of about 20 min was exhibited by benzoate-grown cells. CO_2 release began at the completion of the first oxidation, and ended at the start of the second oxidation. One micromole of CO_2 was released per μmole of 2,3,6-TBA oxidized. This data indicated that a three-step reaction was probably involved in the oxidation of this herbicide.

Measurement of inorganic chloride released during the oxidation of 2,3,6-TBA showed that 1 μmole of chloride was cleaved per μmole of 2,3,6-TBA oxidized. This finding, coupled with manometric data, suggested that a dichlorocatechol could be a product of 2,3,6-trichlorobenzoate oxidation.

Thin-layer chromatography provided evidence for the formation of 3,5-dichlorocatechol from oxidation of 2,3,6-TBA. R_f values in three solvent systems were identical to those of authentic 3,5-dichlorocatechol (chloroform: R_f = 0.17; butanol-benzene-water: R_f = 0.04; butanol-acetic acid-water: R_f = 0.94). Identity of this compound as 3,5-dichlorocatechol was confirmed by infrared spectral analysis. The lead derivative of the chlorocatechol yielded an infrared spectrum which was identical to that of a lead derivative of authentic 3,5-dichlorocatechol (Figure 2).

Oxygen uptake, CO_2 release, chloride release, and 3,5-dichlorocatechol formation were quantitatively measured to determine the pathway of 2,3,6-TBA degradation. As seen in Figure 1, chloride release followed closely the second rise in oxygen uptake, and 3,5-dichlorocatechol formation paralleled the curve of chloride release. One micromole of oxygen was taken up, and 1 μmole each of CO_2, inorganic chloride, and 3,5-dichlorocatechol was produced for each μmole of 2,3,6-TBA oxidized.

The initial oxidation of the herbicide occurred without CO_2 release or chloride cleavage, indicating that hydroxylation took place at either the 4- or 5-position of the benzene ring. The end product of this oxidative pathway, 3,5-dichlorocatechol, could result from an initial hydroxylation at either position by the pathways shown in Figure 3.

Oxygen uptake by the *Brevibacterium* sp. was determined on 2,3,6-, 2,4,5-, and 3,4,5-trichlorophenols. It was proposed that if pathway 1 (Figure 3), involving a one-step oxidation-dehalogenation, was followed, both 2,3,6- and 2,4,5-trichlorophenols should be oxidized but 3,4,5-trichlorophenol should not be due to the lack of a chloro-substituent ortho to the hydroxyl. If pathway 2, involving oxidation ortho to the hydroxyl and dehalogenation meta to the hydroxyl, was followed, both 2,4,5- and 3,4,5-trichlorophenols should be oxidized but 2,3,6-trichlorophenol should not be, due to the lack of a free ortho position.

This isolate oxidized both 2,3,6- and 2,4,5-trichlorophenols, but failed to oxidize 3,4,5-trichlorophenol, indicating that a chlorine ortho to the hydroxyl on the benzene ring was necessary for enzyme action. It appeared therefore that initial oxidation occurred at the 4-position of 2,3,6-TBA and that

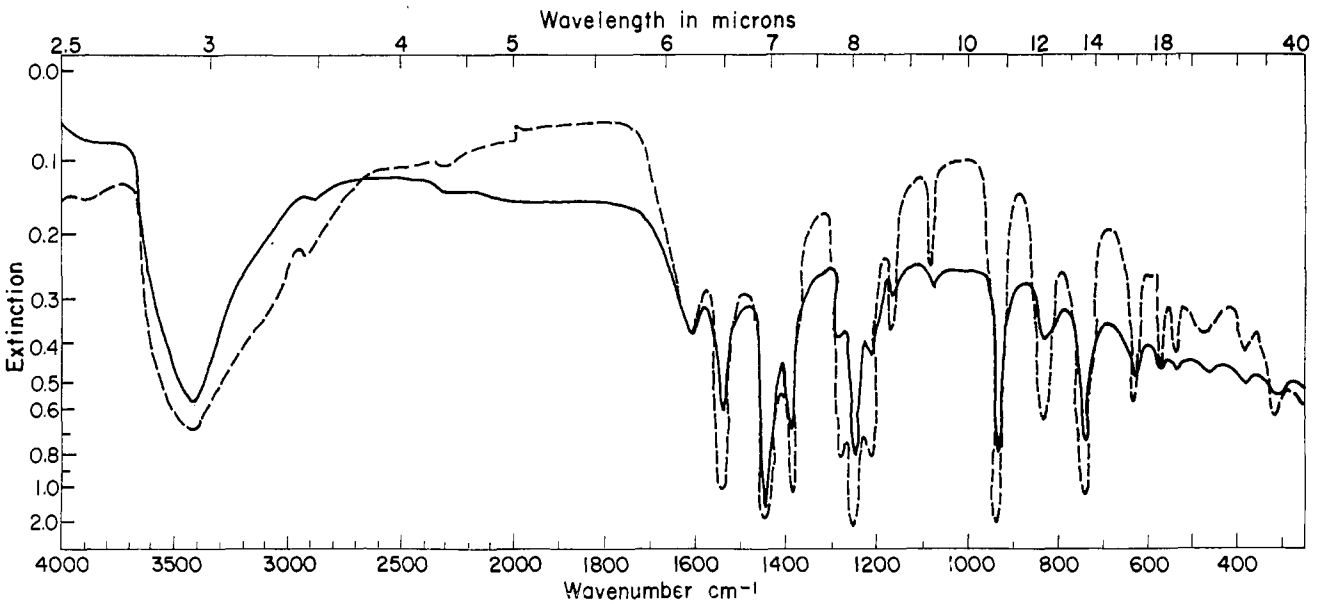

Figure 2. Infrared spectra of authentic lead 3,5-dichlorocatecholate (- - - -) and the lead derivative of the product of cometabolism of 2,3,6-trichlorobenzoate by a *Brevibacterium* sp. (—)

pathway 1 (Figure 3) was followed. This dehalogenation mechanism is consistent with that described by Castro and Bartnicki (1968). The stoichiometry of 3,5-dichlorocatechol formation and 2,3,6-TBA oxidation makes it unlikely that a random dehalogenation mechanism is involved. Also, the varying susceptibility to microbial attack of benzoates carrying halogens in the ortho, meta, and para positions (Alexander, 1964; Alexander and Aleem, 1961; Burger *et al.*, 1962) makes it unlikely that the responsible enzyme is a nonspecific dehalogenase which removes chloride from any position.

Oxygen uptake by whole cells metabolizing benzoate, 2,3,6-trichlorobenzoate, and catechol was reduced to the endogenous respiration level in the presence of 3,5-dichlorocatechol. These results indicated that this dichlorocatechol was toxic to the isolate. Tranter and Cain (1967) had proposed that an accumulation of toxic products could account for the failure of many halogenated aromatic compounds to support growth of bacteria which could oxidize them. It appeared that this explanation could account for the phenomenon of cometabolism of 2,3,6-TBA exhibited by this isolate.

To establish the toxicity of 3,5-dichlorocatechol, 2,3,6-TBA was supplied to the organism at increasing concentrations and chloride release was measured to determine the amount of 2,3,6-TBA oxidized. One micromole of inorganic chloride was released per μmole of 2,3,6-TBA supplied up to a concentration of 0.15 mM trichlorobenzoate. Increasing the concentration of 2,3,6-TBA above this level did not result in an equimolar increase in chloride released. Chloride concentration reached a maximum of 0.15 mM regardless of the concentration of 2,3,6-TBA used. This supported the concept of toxicity of 3,5-dichlorocatechol to the cells.

Cell free enzyme preparations were employed to demonstrate both the inducible nature of the enzymes involved in 2,3,6-TBA oxidation and the enzyme or enzymes inhibited by 3,5-dichlorocatechol. Gibson *et al.* (1968) had shown that halogenated catechols inhibited the enzyme system of *Pseudomonas putida* which catalyzed the incorporation of O_2 into the aromatic nucleus by chelating the iron at the active center of the enzyme. In view of results obtained with whole cell studies, a similar explanation might be applicable in the case of this *Brevibacterium* sp.

The preparation from cells not induced with 2,3,6-TBA prior to sonication failed to oxidize this compound. The induced cell free preparation oxidized 2,3,6-TBA with the uptake of 1 μmole of oxygen per μmole of herbicide both in the presence and absence of 3,5-dichlorocatechol. This established the inducible nature of the trichlorobenzoate oxidase enzymes and showed that these enzymes were not inhibited by 3,5-dichlorocatechol.

Preparations from both induced and noninduced cells oxidized catechol with the uptake of 1 μmole of oxygen per μmole of catechol. This oxidation occurred in the presence as well as the absence of 3,5-dichlorocatechol. Neither the induced preparation nor the noninduced preparation oxidized 3,5-dichlorocatechol. Enzyme specificity and not toxicity appeared to be the explanation of cometabolism of 2,3,6-TBA by this isolate. This is consistent with results obtained with an *Arthrobacter* sp. cometabolizing *m*-chlorobenzoate (Horvath and Alexander, 1970).

The herbicide 2,3,6-trichlorobenzoate is degraded by a cometabolic process to 3,5-dichlorocatechol. The oxidation of this herbicide by the *Brevibacterium* sp. used in this study is catalyzed by inducible enzymes and proceeds through the intermediates 2,3,6-trichloro-4-hydroxybenzoate and 2,3,5-trichlorophenol. The end product of this oxidation, 3,5-dichlorocatechol, accumulates due to specificity of the pyrocatechase enzyme for an unsubstituted catechol. At concentrations of 2,3,6-TBA above 0.15 mM, 3,5-dichlorocatechol accumulates to a concentration which results in production of a toxic environment to the cells This toxicity does not appear to reside at the level of the 2,3,6-TBA oxidizing enzymes, but does result in stoppage of cometabolism by whole cells.

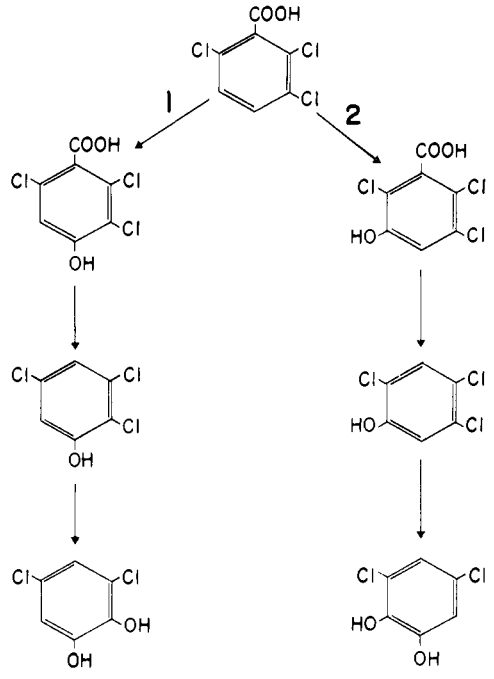

Figure 3. Possible pathways for the formation of 3,5-dichlorocatechol resulting from cometabolism of 2,3,6-trichlorobenzoate by a *Brevibacterium* sp.

This study indicates that the phenomenon of cometabolism is an important factor in the breakdown of pesticides in nature.

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