Cometabolism of products of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) by Pseudomonas putida

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5.8 to 20.4% of the total, while the corresponding mono-N-dealkylated product ranged between 2.5 and 12.5% over a 30-day period (Table II). 2-Chloro-4-amino-6-isopropylamino-*s*-triazine was present at the highest levels between 5 and 10 days after the initial herbicide treatment, while the corresponding mono-N-dealkylated product was detected at the highest levels at 20 days. Although the level of each mono-N-dealkylated atrazine product decreased between 20 and 30 days after the initial exposure, it seems that 2-chloro-4-amino-6-isopropylamino-*s*-triazine is the favored substrate for the second N-dealkylated or possibly the conjugation reaction. The diamino atrazine metabolite resulting from the second dealkylation reaction represented a relatively minor component of the chloroform fraction, but did tend to increase with time through the 30-day experiment (Table II).

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Cometabolism of Products of 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) by *Pseudomonas putida*

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Pseudomonas putida, an organism capable of utilizing diphenylmethane and benzhydrol as sole sources of carbon, cometabolized bis(*p*-chlorophenyl)methane (DDM) to *p,p'*-dichlorobenzhydrol (DBH), *p,p'*-dichlorobenzophenone (DBP), benzhydrol, benzophenone, *p*-chlorophenylacetic acid, and *p*-chlorophenylglycolaldehyde. DBH was converted by the resting cells to DBP, benzhydrol, and benzophenone. No products were detected when the bacterium was incubated with DBP. Bis(*p*-chlorophenyl)acetic acid was cometabolized to DDM, DBH, and DBP. The addition of diphenylmethane to the resting cells did not stimulate the cometabolism of DDM, and no new chlorinated products were detected when the bacteria were provided with both substrates. 1,1,1',1'-Tetra(*p*-chlorophenyl)dimethyl ether was not formed microbiologically from either DDM or DBH.

Cometabolism refers to the metabolism of a substance by a microorganism which is unable to use that compound for energy or as a source of any of the elements required for growth (Horvath and Alexander, 1970). Cometabolism has been employed as a technique for isolating the degradation products of many chlorinated molecules, which are otherwise resistant to biodegradation. With the exception of a few reports, extensive degradation of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has not been reported. Focht and Alexander (1970) demonstrated the extensive transformation of a DDT metabolite in a sequence that involved ring cleavage of bis(*p*-chlorophenyl)methane (DDM), a metabolite generated from DDT. The ring cleavage product was identified as *p*-chlorophenylacetic acid (PCPA). PCPA was also found to be formed by cell-free extracts of *Pseudomonas* sp. (originally classified as *Hydrogenomonas* sp.) incubated first with DDT anaerobically for 4 days and then in the presence of O_2 (Pfaender and Alexander, 1972). Anderson et al. (1970) noted that unidentified water-soluble products were formed from DDT by *Mucor alternans*.

To define more completely the pathways of DDT metabolism, attempts were made to isolate the products formed during the cometabolism of bis(*p*-chlorophenyl)acetic acid (DDA), DDM, *p,p'*-dichlorobenzhydrol (DBH), and *p,p'*-dichlorobenzophenone (DBP) by resting cells of *Pseudomonas putida*. A study was also made of the influence of nonchlorinated analogues on the cometabolism of DDM by *P. putida*.

MATERIALS AND METHODS

Bacterium. The strain of *P. putida* used is capable of utilizing diphenylmethane (DPM) and benzhydrol (BH) as sole sources of carbon. The mineral salts solution used in preparing the growth medium for *P. putida* and for testing the degradability of DDT metabolites was described by Pfaender and Alexander (1973).

Chemicals. The DDT metabolites were obtained from Aldrich Chemical Co., Milwaukee, Wis., except that DDM was purchased from Eastman Organic Chemicals, Rochester, N.Y. 1,1,1',1'-Tetra(*p*-chlorophenyl)dimethyl ether (DCBHE) was synthesized according to the method used for the preparation of its nonchlorinated analogue (Pratt and Draper, 1949).

Respirometry. To obtain resting cells, *P. putida* was

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grown in 1 L of half-strength trypticase soy broth (Baltimore Biological Laboratories) in 2-L Erlenmeyer flasks. The flasks were incubated for 48 h at 30 °C on a rotary shaker operating at 180 rpm. The cells were centrifuged, washed three times in 0.1 M phosphate buffer of pH 7.2, and then incubated in buffer for 12 h on a rotary shaker at 30 °C to reduce the endogenous respiration. The cells were again washed, suspended in buffer, and added to the Warburg flasks. Standard manometric techniques (Umbreit et al., 1972) were used. Each respirometer flask received 1.0 μmol of substrate in 0.5 mL of acetone. The acetone was evaporated in a stream of N_2, and the respirometer flasks then received 2.5 mL of 0.1 M phosphate buffer (pH 7.2), 0.50 mL of the cell suspension (17 mg dry weight), and 0.20 mL of 20% KOH in the center well. The temperature of the water bath was 30 °C.

Preparation of Trimethylsilyl (Me_3Si) Derivatives. An ether solution containing 1 to 2 mg of products or authentic chemicals was placed in small vials covered with screw caps fitted with rubber septa reinforced with nylon and backed with Teflon film (Silli-vials, Applied Science Laboratories, State College, Pa.), and the ether was evaporated under a stream of dry N_2. The samples were redissolved in a few drops of hexanes or pyridine dried over 13X molecular sieves. Regisil-2 (Regis Chemical Co., Chicago, Ill.) was added to the samples at a rate of 0.1 mL/mg of product, and the tightly capped vials were left overnight in the dark at 20 °C or in a 60 °C water bath for 4 h. The reaction mixture was evaporated under a stream of dry N_2, redissolved in ether or hexanes, and analyzed by gas–liquid chromatography.

Gas–Liquid Chromatography. A Varian Aerograph gas–liquid chromatograph, Model 1740-20 (Varian Associates, Palo Alto, Calif.), equipped with a flame ionization detector and two 183 cm × 0.3 cm coiled Pyrex glass columns was used. One of the columns was packed with 3% OV-1 coated on acid washed, dimethylchlorosilane treated 100/120 mesh Gas-Chrom Q (Applied Science Laboratories). The other column was packed with 10% DC-200 coated on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories). The temperature of the OV-1 column was programmed from 110 to 180 °C at a rate of 20 °C/min. The oven temperature for the DC-200 column varied with the experiment. The temperatures of the injector and detector were 225 and 240 °C, respectively. The gas flow rates were 80, 50, and 400 mL/min for N_2, H_2, and air, respectively. Unless otherwise stated, the 3% OV-1 column with the above temperature program was used throughout the studies.

Coupled Gas–Liquid Chromatography–Mass Spectrometry (GC–MS). Mass spectra of products and authentic compounds in solutions were obtained by using a Finnigan-3300 gas chromatograph–mass spectrometer equipped with a Systems-150 data processor. The spectra were scanned at a rate of one scan per second. The ionization voltage was 70 eV. The gas chromatograph was equipped with a 0.3 cm × 305 cm long U-shaped glass column filled with 3% OV-1 coated with 100/120 mesh Gas-Chrom Q. The temperature of the injector was 250 °C, and the column was programmed from 110 to 180 °C at a rate of 20 °C/min.

Products Formed from DDT Metabolites. Resting cells of *P. putida* were obtained by growing the bacteria on a rotary shaker for 3 days at 30 °C in three 2-L flasks, each with 1.0 L of 0.2% glucose–inorganic salts broth containing 0.05% DPM. The cells were washed six times with 0.1 M phosphate buffer (pH 7.2) to free them of residual carbon sources. The reaction mixture consisted

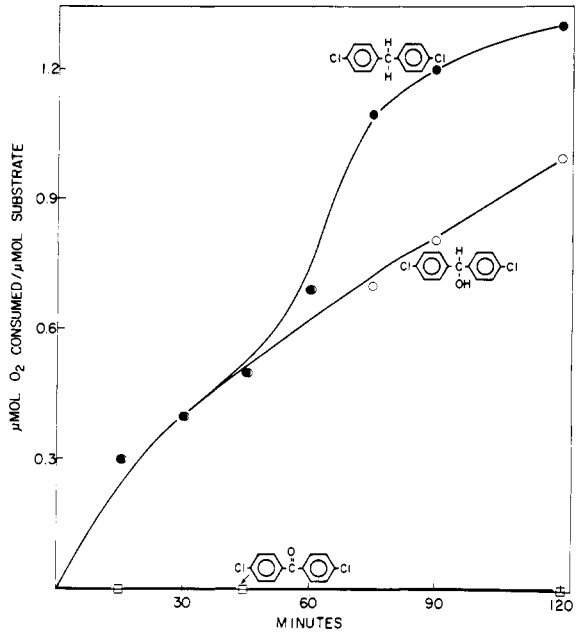

Figure 1. Oxidation of DDT metabolites by resting cells of *Pseudomonas putida*.

of 500 mL of an inorganic salts solution containing 0.05% of DDM, DBH, or DBP, except that 50 mL of 0.1% DDA–inorganic salts solution was used to isolate the products formed from DDA. Nonbiological decomposition of the test chemicals was assessed using the same reaction mixtures incubated without bacteria, and the resting cells were incubated in the substrate-free salts solution in order to differentiate cometabolic products from cellular metabolites liberated by autolysis. Resting cells at an absorbancy of 0.42 at 545 nm were incubated in the reaction medium for 7 days, a period chosen to allow for appreciable product formation, and the reaction was stopped by acidifying the solution to pH 2.0. The reaction mixture was then freed of cells and residual substrate by centrifugation at 4000*g* for 10 min. The supernatant fluids were extracted in separatory funnels, once with one-third volume of hexanes (pesticide grade, Fisher Scientific Co., Rochester, N.Y.) and twice with one-third to one-half volumes of diethyl ether. The extracts were pooled, reextracted with three one-third volumes of an aqueous solution of 5% $NaHCO_3$, and then extracted with distilled water to remove $NaHCO_3$. The solvent fraction presumably contained most of the neutral components. The aqueous phase was acidified to pH 2.0 with HCl and extracted with ether to obtain acidic products. Me_3Si derivatives of both fractions were prepared and analyzed by gas chromatography and GC–MS. The release of chloride ions was detected by the method of Bergmann and Sanik (1957).

Cometabolism of DDM in the Presence of DPM. *P. putida* was grown in 3 L of 0.1% DPM–mineral salts broth on a rotary shaker for 3 days at 30 °C. The cells were washed free of contaminating carbon sources. The reaction mixtures consisted of 1 L of either 0.1% DDM–inorganic salts solution or inorganic salts solution containing 0.05% DPM and 0.05% DDM. The bacteria were added at a rate equivalent to 278 mg of cells (dry weight basis) per liter of solution. The flasks were incubated for 7 days at 30 °C on a rotary shaker, the cells and unutilized solid substrates were separated by centrifugation at 4000*g* for 10 min, and the supernatant fluids were acidified to pH 2.0. The supernatant fluids were continuously extracted for 6 h with anhydrous diethyl ether using a liquid–liquid automatic

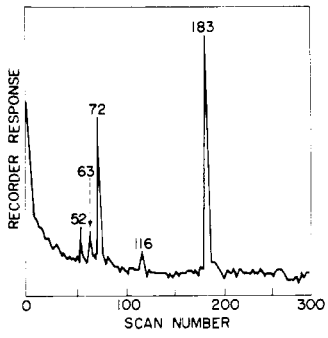

Figure 2. Gas chromatogram of neutral products formed in DDM cometabolism by *Pseudomonas putida*.

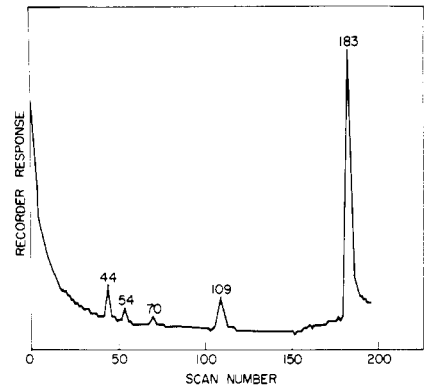

Figure 3. Gas chromatogram of neutral products formed in DBH cometabolism by resting cells of *Pseudomonas putida*.

extractor (Ace Glass Co., Vineland, N.J.) and separated into neutral and acidic fractions by the procedures cited above, except that the supernatant fluids containing both DPM and DDM were extracted as one fraction. Me_3Si derivatives of the products were prepared and analyzed by gas–liquid chromatography and GC–MS.

RESULTS

Degradation Products of DDT Metabolites. The rates of O_2 consumption in respirometer vessels suggested that DDM and DBH but not DBP were cometabolized by *P. putida* (Figure 1). However, DBP was included in subsequent studies because it might be cometabolized by resting cells without giving detectable O_2 utilization.

A gas chromatogram of the neutral extract of reaction mixtures of *P. putida* incubated in 0.05% DDM–salts solution is shown in Figure 2. The compounds at scans 52, 72, 116, and 183 were identified as benzophenone (BP), DDM, DBP, and DBH, respectively. These identifications were based on comparisons of the mass spectra of these compounds with the spectra obtained for authentic compounds. These spectra were identical with published spectra (Stenhagen et al., 1974). The component with a peak at scan 63 was present as a contaminant in the original substrate. Except for BP, these products were also found by Wedemeyer (1967).

The same products were found in the acidic extract, and, in addition, an unidentified compound with a retention time (t_R) of 178 s and BH with a t_R value of 258 s were detected in the acidic extract. Because of their presence in extremely small quantities, none of the products in the acidic extracts was analyzed by GC–MS. The yield of products in the acidic fraction was calculated by measuring the peaks on chromatograms and converting them into quantities of the products from the standard curves prepared for each chemical. The total amount of compounds detected in the acidic fraction was 25.8 μg, and the percent distribution of products was BP, 4.5%; BH, 12.8%; DDM, 45.0%; DBP, 28.8%; and DBH, 8.9%.

The microbial synthesis of BH and BP could not be confirmed by measuring chloride release because of the small amount of chloride ions generated during the cometabolism of DDM. BH and BP were not present at zero time, in resting cells incubated in the substrate-free salts solution, or in suspensions of cells boiled for 15 min prior to incubation with DDM.

The products in the neutral extract of *P. putida* incubated with DBH are indicated in the gas chromatogram presented in Figure 3. The mass spectra obtained for scans 44, 54, 109, and 183 were identical with the spectra obtained for authentic BP, BH, DBP, and DBH, respectively. These spectra were also identical with published spectra (Stenhagen et al., 1969, 1974). The product at scan 70 was not identified. The t_R values of these compounds were identical with those of authentic compounds. The t_R values of BP, BH, DBP, and DBH were 236, 265, 556, and 649 s, respectively.

An additional product was detected in the acid fraction derived from cells incubated with DBH. The mass spectrum of its Me_3Si derivative is shown in Figure 4. Based on the finding of major fragmentation peaks at m/e 213 and 215 and because of the expected fragmentation pattern for the Me_3Si derivative of *p*-chlorophenylglycolic acid, the product was suspected to be the Me_3Si ether of *p*-chlorophenylglycolic acid. However, the mass spectrum obtained for the Me_3Si derivative of authentic *p*-chlorophenylglycolic acid did not coincide with the mass spectrum of the unknown.

Me_3Si derivatives of both neutral and acidic fractions of suspensions of *P. putida* incubated with DBP were analyzed by gas–liquid chromatography. No metabolites were detected in these fractions, and DBP was completely recovered from the reaction solutions.

DDM, DBH, and DBP with t_R values of 385, 649 and 556 s, respectively, were found as products of the cometabolism of DDA by the resting cells. These t_R values were identical with the values obtained for the authentic compounds. Their mass spectra were also identical with the spectra obtained for authentic chemicals.

Cometabolism of DDM in the Presence of DPM. The experiment was designed to determine whether a readily metabolizable analogue might enhance the cometabolism of chlorinated products known to be formed biologically from DDT. The components were extracted from a mixture containing resting cells of *P. putida* and either a DDM–inorganic salts solution or a salts solution containing both DDM and DPM. The acid extract of the reaction mixtures containing cells and DDM had two products. The mass spectrum of the major component was identical with the spectrum obtained for *p*-chlorophenylglycolaldehyde by Pfaender and Alexander (1972). The t_R values of this compound were 105 s on a 3% OV-1 column programmed from 140 to 200 °C at a rate of 10 °C/min and 229 s on a 10% DC-200 column maintained at 140 °C. The compound gave a silver mirror with Tollen's reagent (Cheronis et al., 1965), in confirmation of the identity of *p*-chlorophenylglycolaldehyde. The yield of *p*-chlorophenylglycolaldehyde was estimated by comparing the peak area for the aldehyde with peak areas obtained for authentic PCPA; on this basis, 2.3 mg of *p*-chlorophenylglycolaldehyde was produced in the cometabolism of 250 mg of DDM. The extract also contained 15.8 μg of PCPA, which was detected as the Me_3Si derivative and identified by cochromatography of the Me_3Si derivatives of authentic PCPA and of the bacterial product.

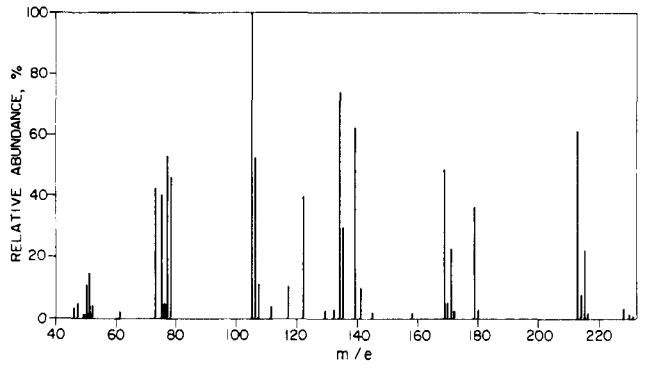

Figure 4. Mass spectrum of Me_3Si derivative of product in acidic extract of resting cells of *Pseudomonas putida* incubated with DBH.

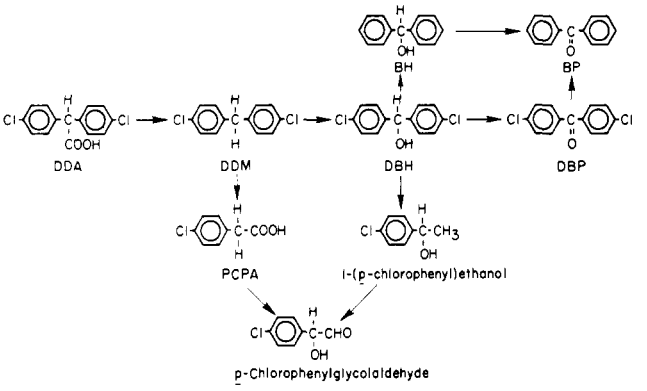

Figure 5. Postulated pathway for the metabolism of DDA, DDM, DBH, and DBP.

The Me_3Si derivatives of the products in neutral extract of the DDM–salts solutions incubated with resting cells of *P. putida* were analyzed by gas–liquid chromatography using a 10% DC-200 column. The column was maintained initially at 110 °C for 1 min, and then the temperature was raised to 175 °C at a rate of 20 °C/min. The peaks on the gas chromatogram with t_R values of 129, 243, 493, 564, 718, and 932 s were identified as PCPA (identified as the Me_3Si ether), *p*-chlorophenylglycolaldehyde, an isomer of DDM, DDM, DBP, and DBH, respectively. The products were identified by their mass spectra obtained by GC–MS. Two unknown products with t_R values of 357 and 1142 s were also detected in the neutral extract. None of these products was evident in the bacteria-free salts solution amended with DDM or in the zero-hour samples containing both cells and DDM.

The reaction mixture containing both DPM and DDM was extracted and analyzed as one fraction containing both neutral and acidic components. Three products were found, and these were identified as phenylglycolic acid (identified as its Me_3Si ether), DBH, and DBP by comparing their mass spectra with the spectra obtained for authentic compound. The spectra were also identical with published spectra (Stenhagen et al., 1969, 1974). Thus, the addition of DPM did not lead to the formation of any new metabolites from DDM. No products were detected in the control flasks maintained to assess for nonbiological degradation of both DDM and DPM or in zero-hour samples of the treatments.

Possible Formation of DCBHE. DCBHE was suspected to be formed from DDM, as its nonchlorinated analogue (1,1,1′,1′-tetraphenyldimethyl ether) is synthesized during the metabolism of DPM by *P. putida* (Subba-Rao and Alexander, 1977). However, DCBHE was not found in the ether extracts of solutions containing resting cells of *P. putida* incubated with DDM or DBH. DCBHE had a retention time of 2050 s on a 3% OV-1 column maintained at 250 °C.

DISCUSSION

The possible reactions involved in the conversions resulting from the activities of *P. putida* are presented in Figure 5. The initial products formed during the cometabolism of DDA were suggested by Wedemeyer (1967) on the basis of his studies of *Klebsiella pneumoniae*. The finding that BH and BP were formed from DDM and DBH indicates that a dehalogenation had occurred. No such nonchlorinated molecules have been detected during the metabolism of DDT in nature, but this is not unexpected because, once formed, they would likely be degraded rapidly. The postulated conversion of DBP to BP has not been demonstrated, but it is included because of the analogy to DBH dehalogenation.

DDM was also converted to PCPA, which is a possible precursor of *p*-chlorophenylglycolaldehyde. Alternatively, *p*-chlorophenylglycolaldehyde may be generated from DBH should the latter undergo ring cleavage to yield 1-(*p*-chlorophenyl)ethanol, the oxidation of which might lead to the formation of the glycolaldehyde.

Stimulation of DDT metabolism in model marine ecosystems by the addition of structural analogues has been previously reported (Juengst and Alexander, 1975). However, no such stimulation of DDM metabolism was observed with resting cells of *P. putida* that were provided with DPM as a source of carbon and energy. PCPA and *p*-chlorophenylglycolaldehyde, which were detected in the solutions containing only DDM, were even not formed when the cells of *P. putida* were provided with both DPM and DDM. These studies thus suggest that the cells do not attack the chlorinated molecules when they are provided with readily utilizable analogues.

The present study has demonstrated the microbial formation of substances previously unrecognized as products of the biological breakdown of metabolites of DDT. Additional work is required to establish which are indeed released by microbial communities acting on the insecticide in nature.

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