

Products Formed from Analogues of 1,1,1-Trichloro-2,2-Bis(*p*-Chlorophenyl)Ethane (DDT) Metabolites by *Pseudomonas putida*

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Cultures of *Pseudomonas putida* growing in solutions with diphenylmethane as sole carbon source formed 1,1,1',1'-tetraphenyldimethyl ether. The product was identified by gas chromatography, mass spectrometry, and infrared and nuclear magnetic resonance spectrometry. The formation of benzophenone, benzhydrol, and phenylglycolic acid was established by gas chromatography and mass spectrometry. Similar techniques also revealed that phenylacetic acid was a major metabolite. Resting cell suspensions converted benzhydrol to phenylglycolic acid and products tentatively identified as hydroxybenzhydrols and a hydroxybenzophenone. Cell suspensions of the bacterium also converted the tetraphenyldimethyl ether to benzhydrol and benzophenone. Possible pathways for the degradation of these analogues of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) metabolites are discussed.

Microorganisms capable of utilizing 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) as sole source of carbon have yet to be isolated. However, various species convert DDT to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene, and *p,p'*-dichlorobenzophenone (DBP) (7, 9, 18). The further metabolism of DBP has not yet been observed, and DBP seems to be resistant to microbial degradation (5, 8). The inability to isolate microorganisms able to grow by using DBP or dichlorinated diphenylmethanes as carbon sources has hindered establishment of the pathway of DDT degradation.

One means of obtaining information on the possible pathways of DDT metabolism is to characterize the pathway of degradation of nonchlorinated analogues of DDT and its metabolites. Thus, the model substrates diphenylmethane (DPM), bis(*p*-chlorophenyl)methane (DDM), and 1,1-diphenyl-2,2,2-trichloroethane were found to be converted to phenylacetic acid (PA), *p*-chlorophenylacetic acid, and 2-phenyl-3,3,3-trichloropropionic acid by resting cells of *Pseudomonas* sp., an isolate originally classified as a strain of *Hydrogenomonas* (6).

The present investigation was designed to establish the pathways for the metabolism of nonchlorinated analogues of DDT metabolites such as DDM, *p,p'*-dichlorobenzhydrol, and

DBP. For this purpose, DPM, benzhydrol (BH), and benzophenone (BP) were used as substrates.

MATERIALS AND METHODS

Organism. *Pseudomonas putida* was isolated from enrichments containing 0.1% DPM inoculated with a suspension of Hudson-Collamer silt loam. The salts solution used in the enrichments and in all other studies has been described by Pfaender and Alexander (9).

Respirometry. *P. putida* was grown for 48 h on a rotary shaker at 30°C in 1 liter of half-strength Trypticase soy broth (Baltimore Biological Laboratories) in 2-liter Erlenmeyer flasks. The cells were collected by centrifugation and washed three times in 0.1 M phosphate buffer, pH 7.2. The cells were incubated in buffer for 6 to 12 h on a rotary shaker at 30°C to reduce the endogenous respiration. The cells were again washed, resuspended in buffer, and added to the respirometer flasks. Standard manometric procedures (16) were used. Each Warburg flask contained 1.0 μmol of substrate in 0.5 ml of acetone, the acetone being evaporated in a stream of N₂, 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 was 30°C.

Chemicals. DDT analogues of highest purity were obtained from Aldrich Chemical Co., Milwaukee, Wis. 1,1,1',1'-Tetraphenyldimethyl ether (BHE) was prepared by the method of Pratt and Draper (10).

Thin-layer chromatography. Thin-layer chromatography (TLC) was carried out with chromagram sheets (20 by 20 cm) coated with silica gel containing

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a fluorescent indicator (Eastman) exciting at 254 nm. For preparative TLC, 2-mm-thick silica gel with ultraviolet 254 indicator coated on glass plates (20 by 20 cm) (Brinkmann Instrument Co., Westbury, N.Y.) was used. The plates were developed with hexane-ether-acetic acid (100:10:5), and the spots were detected by exposing the plates to an ultraviolet (UV) (254 nm) lamp. The reagents for characterizing unknown compounds on TLC plates were prepared as described by Stahl (12).

Preparation of trimethylsilyl (TMS) derivatives. Ether solutions containing 1 to 2 mg of products were placed in small vials covered with screw caps fitted with rubber septa reinforced with nylon and backed with a Teflon film. The ether was allowed to evaporate under a stream of dry N_2 . The samples were dissolved in a few drops of hexanes or pyridine dried over $13\times$ molecular sieves (Fisher). Regisil-2 (Regis Chemical Co., Chicago, Ill.) was added to the samples at a rate of 0.1 ml/mg of chemical, and the tightly capped vials were left overnight in the dark at 20°C or for 4 h at 60°C. The reaction mixture was evaporated under a stream of N_2 , redissolved in ether or hexanes, and injected onto the column of a gas-liquid chromatograph.

Gas-liquid chromatography. A Varian aerograph gas-liquid chromatograph, model 1740-20 (Varian Associates, Palo Alto, Cal.), equipped with a flame ionization detector and containing a (183 cm by 0.3 cm) coiled Pyrex glass column packed with 3% OV-1 coated on acid-washed, dimethylchlorosilane-treated, 100/120 mesh Gas-Chrom Q (Applied Science Laboratories) was used. The temperature of the column was programmed from 110 to 180°C at a rate of 20°C/min. 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 carrier gas (N_2), H_2 , and air, respectively.

Spectrometry. Mass spectra of crystalline solids were obtained with an Associated Electrical Industries (Manchester, England) mass spectrometer, model MS 9, using a direct probe inlet system. Mass spectra of many products in solution were obtained with Finnigan-3300 gas chromatograph-mass spectrometer (GC-MS) equipped with a Systems-150 data processor. The sample spectra were scanned at a rate of one scan per second. The ionization voltage in the mass spectrometers was 70 eV. The gas chromatograph of the coupled GC-MS system was equipped with a U-shaped glass column (0.3 by 305 cm) filled with 3% OV-1 coated on 100/120 mesh Gas-Chrom Q. The injector temperature was 250°C, and the column was programmed either from 150 to 200°C at a rate of 10°C/min or from 110 to 180°C at 20°C/min.

UV absorption spectra of chemicals in 95% ethanol or hexane were obtained with a Beckman spectrophotometer, model DB-G. Infrared (IR) spectra were obtained on samples prepared as KBr mini-pellets using a Beckman IR spectrophotometer, model IR-10. Nuclear magnetic resonance (NMR) spectra were obtained on a miNiMaR instrument (Japan Electron Optics Laboratories, Tokyo) using 10- to 15-mg samples dissolved in 0.4 ml of $CDCl_3$ (Mallinckrodt). Tetramethylsilane was the internal

reference. The spectra were obtained at an applied field frequency of 60×10^6 cps.

Products from DPM. To obtain neutral products, the bacterium was grown at 30°C on a rotary shaker for 4 days in 1 liter of 0.5% (vol/vol) DPM-inorganic salts broth in a 2-liter Erlenmeyer flask. The formation of phenols and catechols was determined by the methods of Swain and Hillis (15) and Evans (3), respectively. The cells were removed from the culture by centrifugation at $4,100 \times g$ for 10 min, and the neutral products were extracted three times from the supernatant fluid with one-fifth volumes of hexanes. The extracts were pooled, dried over anhydrous Na_2SO_4 , concentrated in a flash evaporator to approximately 1.0 ml, and dried in a stream of N_2 . The resulting product was recrystallized from ethanol, and its purity was checked by TLC using 100- μ m silica gel plates and 10% acetone-hexane as the solvent system. The R_f values of the products were determined simultaneously by TLC.

To isolate other products of DPM metabolism, the bacterium was grown in 1.0 liter of 0.1% DPM-salts broth for 4 days on a rotary shaker, the cells were collected by centrifugation, they were washed three times with phosphate buffer (pH 7.2) to remove residual DPM, and these cells were incubated in 100 ml of the buffer for 12 h to deplete their endogenous carbon reserves. Into each of three 2-liter flasks containing 1.0 liter of sterile 0.5% (vol/vol) DPM-salts broth was added a quantity of cell suspension equivalent to 150 mg of dry weight. After 2 days of growth, the culture was acidified to pH 2.0 and extracted with diethyl ether-isopropyl alcohol (10:1) for 6 h. The extract thus obtained was concentrated to approximately 100 ml and was extracted with three one-third volumes of 5% $NaHCO_3$. The solvent fraction presumably contained most of the neutral components. The aqueous phase was acidified to pH 2.0 with HCl and reextracted with ether to obtain the acidic products. The neutral and acidic fractions were subjected to TLC with 10% acetone in hexane and hexane-ether-acetic acid (100:10:5) as solvent systems, respectively. The components of the acidic portion of the extract were separated on thick-layer plates using hexane-ether-acetic acid (100:10:5) as the solvent system. The products obtained in large quantities were subjected to melting point determinations and UV and IR spectrometry, and mass spectra of their TMS derivatives were obtained.

Products from BH. The bacterium was grown on a rotary shaker for 3 days at 30°C in three 2-liter flasks, each containing 1 liter of 0.1% DPM-salts broth. The cells were collected, washed free of residual DPM with 0.1 M phosphate buffer (pH 7.2), incubated in 100 ml of the buffer for 12 h, again washed, and resuspended in the buffer. To three 2-liter flasks, each containing 1.0 liter of 0.2% BH-salts broth, 125 mg of cells (dry weight) was added, and the flasks were incubated at 30°C on a shaker. After 4 days the cells were removed by centrifugation, and the supernatant fluids were acidified to pH 2.0 and extracted for 6 h with diethyl ether-isopropyl alcohol (20:1). The ether extract was concentrated to 100 ml and extracted six times with one-fourth volumes of 5% $NaHCO_3$. The solvent portion containing

the neutral products was concentrated to 5 ml in a flash evaporator. The NaHCO_3 extract was adjusted to pH 2.0 and extracted four times with one-third volumes of ether. The ether portions were pooled, concentrated, and dried over anhydrous Na_2SO_4 .

TMS derivatives of the neutral and acidic products were prepared and analyzed by gas-liquid chromatography. Only 10% of the acidic fraction was silylated, and products in the remainder were separated by preparative layer chromatography. The acidic fraction was streaked as a band on several thick-layer plates and developed in hexane-ether-acetic acid. The products were scraped off the plates and eluted with absolute ethanol. The major component was concentrated and crystallized from ethanol. For the analysis of the acidic and neutral fractions by coupled GC-MS, the temperature of the column was kept at 150°C for 1 min and programmed to 200°C at a rate of 10°C/min. The mass spectra of all detectable components were compared with the spectra of authentic compounds.

Products of BP and BHE metabolism. The bacterium was grown for 3 days at 30°C in 3 liters of 0.2% glucose-inorganic salts broth containing 0.05% of DPM. The cells were washed free of residual carbon sources by the procedures described above. The reaction mixtures consisted of 500 ml of 0.1% BP-salts solution and 100 ml of 0.1% BHE salts solution. The washed cells were added to the reaction mixture to a final absorbancy of 0.42 at 545 nm and incubated for 7 days. The liquid was then acidified to pH 2.0 and centrifuged at $4,100 \times g$ for 10 min. The supernatant fluids were then extracted, separated into neutral and acidic fractions, and analyzed by gas chromatography and GC-MS.

RESULTS

Oxidation of DPM, BH, and BP. *P. putida* grew readily on agar plates containing DPM or BH as the sole carbon source. The utilization of BH was indicated by the formation of clearing zones around the bacterial colonies growing on the agar. The resting cells obtained on Trypticase soy broth were able to oxidize DPM, BH, and BP in respirometers without any lag period (Fig. 1). The extent of oxidation suggested appreciable degradation of the three compounds.

Production of BHE. Small amounts of phenols were detected in the supernatant fluids of *P. putida* cultures grown for 2 days in DPM-salts broth, but they disappeared after the second day of incubation. Catechols were not detected.

Colorless rhombic crystals were obtained from a neutral extract derived from the culture supernatant by crystallizations with ethanol. The yield of crystalline product was 155 mg/liter of 0.5% (vol/vol) DPM-salts broth, which amounts to 3.1% of the DPM added. Only one component was evident when the product was examined on thin-layer plates developed in 10% acetone-hexane, and its R_f value (0.43) did not

correspond to the R_f values of BH (0.20), BP (0.37), or PA (0.03). The crystals melted at 109 to 110°C, whereas the melting points of authentic BH and BP were 67.0 to 67.5 and 47 to 48°C, respectively.

The mass spectrum of the product is given in Fig. 2. The fragmentation pattern of the compound with most abundant peaks at m/e 77, 105, 167, 168, and 183 indicates a benzhydrol-like structure, except that the peak at m/e 184 representing the molecular ion (M^+) for BH was not detected in the mass spectrum. The spectrum also suggests M^+ was either 183 or was too unstable to be detected. If the M^+ ion was 183, the molecule must have a nitrogen atom (11), but the substrate contained no nitrogen. The product was not diphenylamine, whose melting point was 53 to 54°C. The compound was then

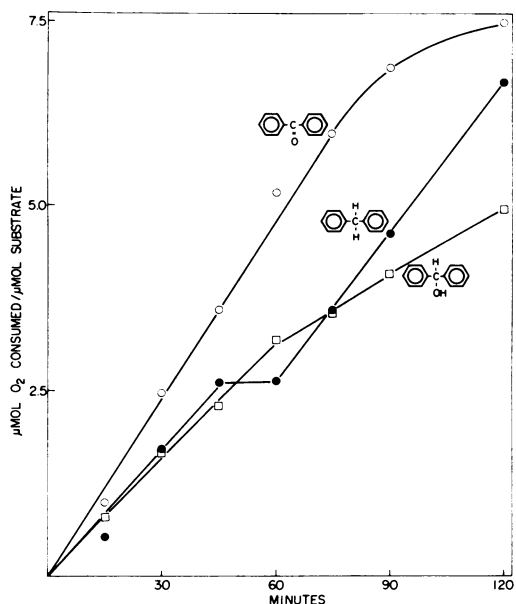


FIG. 1. Oxidation of DPM, BH, and BP by *P. putida*.

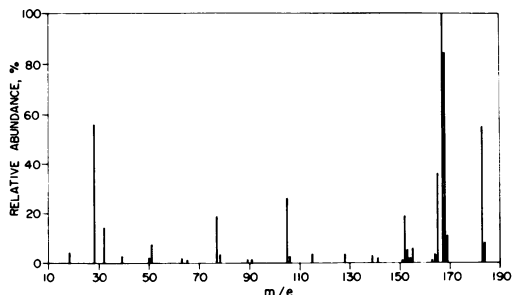


FIG. 2. Mass spectrum of a product of DPM metabolism.

suspected to be BHE, a compound melting at 109 to 110°C (17) and with a presumably similar fragmentation pattern. BHE was then synthesized, and the mass spectra of the synthetic chemical and the bacterial product were found to be identical. It is noteworthy that this compound was not produced when high cell densities (equivalent to 150 mg [dry weight] per liter) were used.

Further confirmation of the identification was then sought. Mixed melting point determinations of the synthetic and biologically formed BHE revealed that the melting point was not depressed. Both authentic BHE and the bacterial product had absorption maxima at 260 and 228 nm in hexane. The IR of biological and synthetic BHE were identical and showed bands at 3,150, 1,610, 1,500, 1,450, 1,340, 1,290, 1,185, 1,080, 1,050, 1,030, 1,010, 920, 765, 735, and 695 cm^{-1} ; a similar spectrum was obtained by Wang (17). The NMR spectrum of the bacterial product (Fig. 3) was identical to that of the synthetic chemical, the spectrum of which is not shown. Integration of the peaks at τ CDCl_3 2.55 and 4.40 revealed that they were in a ratio of 10.5:1, a value corresponding to the 10:1 ratio of the number of protons on the rings to the number on the dimethyl-ether linkage. As expected, the ether showed nonpolar characteristics on a 3% OV-1 column in the gas chromatograph, and a peak for BHE was detected at 1,525 s at a column oven temperature of 205°C. The retention times of authentic BHE, BH, and

BP were 1,525, 210, and 195 s, respectively.

The production of BHE by biological means was confirmed by analyses of various controls. Thus, BHE was not detected in uninoculated media containing DPM or 0.5% BH, and it was not present in the DPM-free salts solution receiving the bacteria and incubated for the same period of time. BHE was also not present at zero time in solutions containing cells and DPM.

Other products of DPM metabolism. The neutral fraction obtained from 3 liters of reaction mixture was concentrated to 8 ml of a viscous solution containing neutral components, which accounted for 53.3% by weight of the DPM added. A dilute sample of the neutral fraction was analyzed by GC-MS, and the gas-chromatographic trace is presented in Fig. 4. The mass spectra of scans 9, 17, and 31 of Fig. 4 were identical with the spectra for authentic DPM, BP, and BH, respectively (13, 14). By measuring the peak areas in the gas chromatograms, it was calculated that 46.6% of the DPM still remained in the reaction mixture after 2 days of incubation and that the yields of BP and BH were equivalent to 4.1 and 8.4% of the DPM metabolized.

The components of the acidic fraction were separated on thick-layer silica gel plates, and bands with R_f values of 0.04, 0.27, and 0.35 were obtained. These three fractions were scraped off the plates, eluted with ethanol, and concentrated by flash evaporation. The fraction of R_f 0.27 was the major component, and it gave

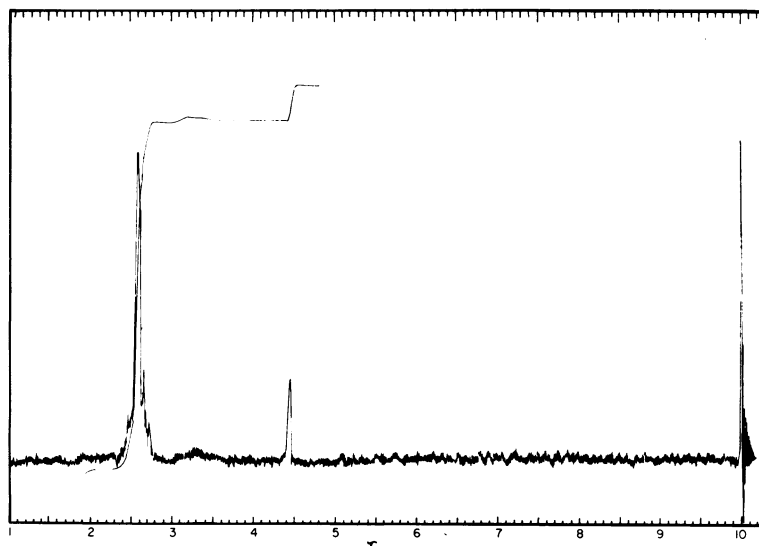


FIG. 3. NMR spectrum of a neutral product formed from DPM.

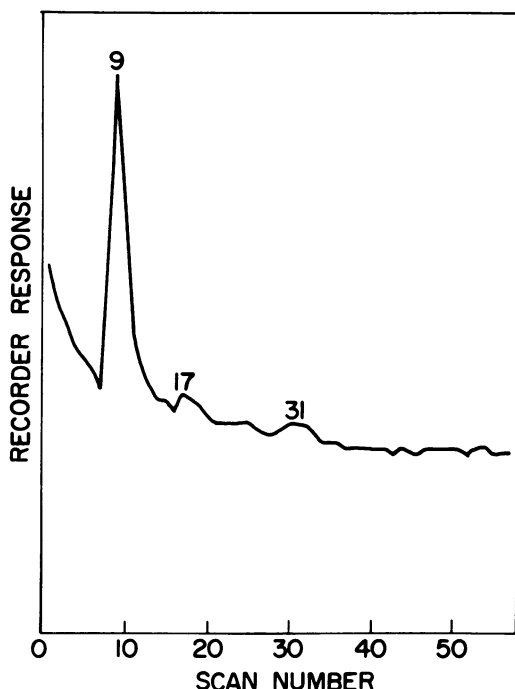


FIG. 4. Gas chromatogram of neutral products generated in DPM metabolism by *P. putida*.

white crystalline plates from ethanol. These crystals melted at 77 to 78°C. Gas-chromatographic analysis (column temperature of 170°C) of TMS derivatives of this component gave a single peak of retention time (R_t) 59 s. The R_t values, R_f values on preparative-layer plates, and melting points of the major component and authentic PA were identical. The UV absorption maxima at 260 and 266 nm and the IR absorption bands at 3,400 to 2,500, 1,730 to 1,640, 1,390, 1,330, 1,250 to 1,170, 920 to 850, 735, 680 to 650, and 580 cm^{-1} of the biological product and authentic PA were identical. These data confirm that the major acidic component was PA. A total of 1.282 g of PA was recovered, which is equivalent to an 18.2% conversion of the metabolized DPM.

The mass spectrum of the TMS derivative of the component with an R_t value of 0.35 was identical to the spectrum obtained for the TMS derivative of authentic phenylglycolic acid. The fragmentation patterns of the biological and the authentic TMS derivative of phenylglycolic acid were identical to that reported by Stenhagen et al. (14). The R_t values of the TMS derivative of the product from DPM and of authentic phenylglycolic acid were identical (212 s).

A total of 63.8% of the DPM initially added

was recovered as DPM, BP, BH, and PA. The yield of phenylglycolic acid was extremely small. None of these products was detected in uninoculated DPM-containing medium or in the DPM-free salts solution receiving the resting cells.

Products from BH. After growth of *P. putida* in BH-salts broth for 12 h, the medium was straw yellow, and the color remained until the end of the incubation period. Phenols were produced and persisted during the incubation period, but catechols were not found.

The neutral fraction of the extract of this culture was silylated, and the derivatives so obtained were analyzed by GC-MS. The gas-chromatographic trace obtained is presented in Fig. 5. Six products were detected by their distinct mass fragmentation patterns. The mass spectrum of the peak at scan no. 6 was identical to the spectrum of the TMS derivative of authentic phenylglycolic acid (14). A small amount of this acidic product was extracted together with the neutral products. The product at scan 8 could not be identified, but its mass spectrum had a base peak at m/e 117 indicating a silylated carboxylate ion and a fragment ion at m/e 145, which might be a silylated glyoxylate ion. The compound represented by scan 15 was identified as unutilized BH by comparing its R_t value (265 s) and its mass spectrum with the TMS derivative of authentic BH. The mass

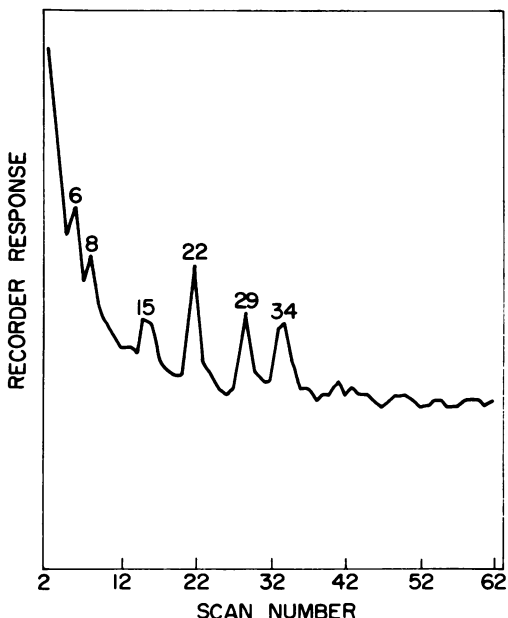


FIG. 5. Gas chromatogram of neutral products of BH metabolism by *P. putida*.

spectrum had peaks for M^+ ion (m/e 256), loss of phenyl ring (m/e 179), loss of $-OTMS$ (m/e 167, base peak), and TMS (m/e 73). Four milligrams of BH was recovered from the neutral extract, a small yield compared to the total of 6 g of BH added to the medium.

The mass spectrum of scan 22 (Fig. 6) was tentatively identified as a hydroxylated BP. The M^+ ion was detected, but the mass spectrum did not coincide entirely with the spectrum of the TMS derivative of authentic *p*-hydroxybenzophenone. The product thus might be *o*- or *m*-hydroxybenzophenone. The mass spectra of scans 29 and 34 (Fig. 7) were tentatively identified as hydroxylated benzhydrols, but authentic hydroxylated benzhydrols were not prepared. The total amount of products detected in the neutral fraction, expressed as BH, was 17 mg.

Products with R_f values on TLC plates of 0.33 and 0.20 were obtained from the acidic fraction. The compound with greater mobility was the major component. The major metabolite responded positively with bromocresol green, indicating that it was acidic. This compound was separated from the acid fraction by preparative-layer chromatography. Whitish crystalline plates melting at 132 to 135°C were obtained when this metabolite was crystallized from ethanol. The TMS derivatives of components of the acidic fraction were analyzed by GC-MS. Two products with similar mass fragmentation patterns were obtained. The mass spectrum of the major component was identical to the spectrum of the TMS derivative of authentic phenylglycolic acid and also with the spectrum reported by Stenhagen et al. (14). The UV absorption maxima at 228 and 260 nm and the IR absorption bands at 3,400 to 3,380, 3,100 to 2,500, 1,740 to 1,680, 1,300 to 1,220, 1,050, 890 to 840, 700 to 660, and 530 to 450 cm^{-1} of the biologically produced and authentic phenylglycolic acid were identical. The minor component was not identified. None of these products was detected in uninoculated BH-mineral salts broth or in the BH-free salts solution contain-

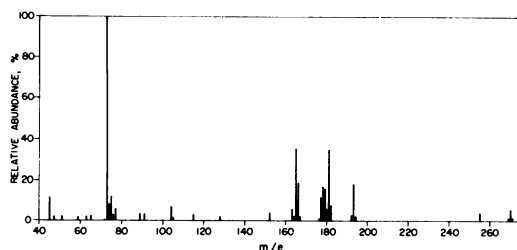


FIG. 6. Mass spectrum of scan 22 of previous figure.

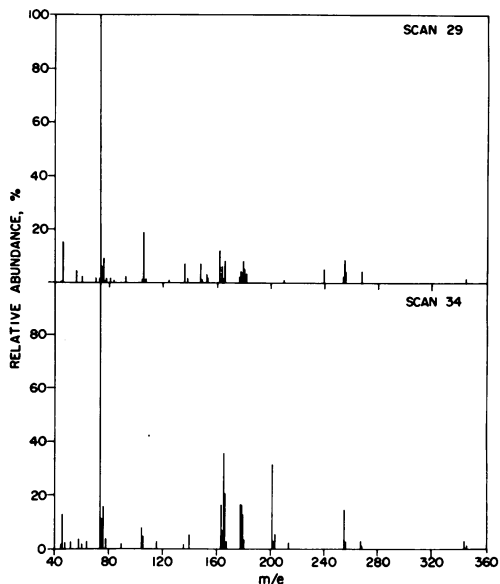


FIG. 7. Mass spectra of scans 29 (top) and 34 (bottom) of Fig. 5.

ing the bacterium. The yield of phenylglycolic acid from 3 liters of broth was 1.695 g, which is equivalent to 34.2% of the added BH. During the metabolism of BH, the yield of *P. putida* cells increased by 185 mg (dry weight) per 3 liters of medium.

BHE metabolism. BHE was cometabolized by resting cells of *P. putida* to BH and BP. The identification of these products was accomplished by comparing their mass spectra with those of authentic BH and BP (13, 14). For gas chromatography, the column was programmed from 110 to 180°C at a rate of 20°C/min; after 5 min, the temperature was raised at a rate of 20°C/min to 200°C. The retention times of BP, BH, and BHE were 237, 263, and 1,850 s, which were identical with the retention times of the authentic compounds. From the chromatograms, the yields were calculated to be 0.5% of BP, 23.3% of BH, and 76.2% of BHE. None of these products was found in cell suspensions incubated in the absence of the substrate, in uninoculated medium, or in samples taken at zero time.

DISCUSSION

During the metabolism of DPM by *P. putida*, BHE was formed. This is the first report of the microbial production of a complex ether of this sort. The lack of accumulation of BHE when high cell densities were used was probably a result of the rapid conversion of DPM to BH, PA, or other products by the large number of

cells added to the medium. By contrast, the amount of cells added to solutions in which BHE was produced may have been too low to deplete rapidly the large quantity of DPM added. BHE may be synthesized by the condensation of DPM with a benzhydrylic radical formed in DPM metabolism. The formation of the benzhydrylic radical may be enzymatic, and the subsequent condensation reaction may be nonenzymatic. Alternatively, BHE might be generated by the condensation of 2 mol of BH with the enzymatic removal of 1 mol of water.

The data suggest that *P. putida* may metabolize DPM by three pathways (Fig. 8): formation of BHE, oxidation to BH and BP, and ring cleavage with the production of PA. The major routes of DPM and BH metabolism appear to involve synthesis of PA and phenylglycolic acid. BHE is either directly converted to BH and BP or the latter may be formed after the ether is converted by a reversible reaction back to DPM. The oxidation of DPM to BH and BP is analogous to the pathway of metabolism of DDM by *Klebsiella pneumoniae* proposed by Wedemeyer (18).

In view of the finding of PA and phenylglycolic acid formation in the metabolism of DPM by *P. putida* and the identification by Focht and Alexander (4) of phenylglyoxylic acid and benzoic acid during the degradation of DPM by *Pseudomonas* sp., a new pathway for the metabolism of PA may be proposed (Fig. 8). The metabolism of PA might thus involve the formation of either homogentisic acid or phenylglycolic acid. Homogentisic acid degradation has received considerable attention (2) as has the metabolism of benzoic acid (1). The phenylglycolic acid may then be oxidized to phenylglyoxylic and ultimately benzoic acids.

The data suggest the possibility for the for-

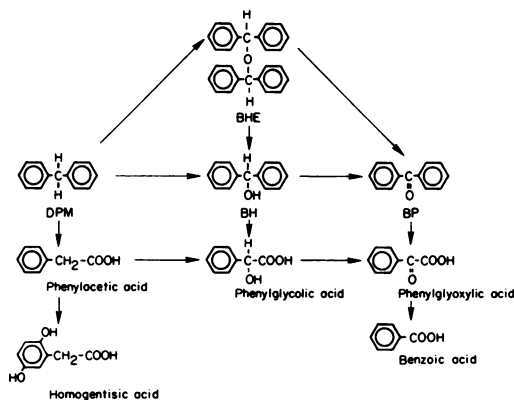


FIG. 8. Proposed pathway for the metabolism of DPM, BH, and BP by *P. putida*.

mation in nature of the chlorinated analogue of BHE. If it is formed, the problem with DDT may be aggravated because this analogue, 1,1,1',1'-tetra(*p*-chlorophenyl)dimethyl ether, is nonpolar and might accumulate in food chains. It is also possible that DDT is converted to *p*-chlorophenylacetic acid, *p*-chlorophenylglycolic acid, and *p*-chlorophenylglyoxylic acid, these compounds being the chlorinated analogues of the bacterial products identified in the present study. *p*-Chlorophenylacetic acid was earlier reported to be formed from DDM (6). These chlorinated products of cleavage of one of the two rings of the diphenyl compounds are probably degraded microbiologically at reasonably rapid rates.

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