Bacterial Degradation of Diphenylmethane, a DDT Model Substrate

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A strain of *Hydrogenomonas* was isolated by elective culture in a solution with diphenylmethane, an analogue of DDT, as the sole carbon source. Constitutive enzymes effected the oxidation and fission of one of the benzene rings of diphenylmethane, and phenylacetic acid was found as a major degradation product. Small amounts of phenylglyoxylic and benzoic acids were also generated from diphenylmethane by the bacterium. Phenylacetic acid, which contains the second benzene ring of diphenylmethane, was metabolized by inducible enzymes.

The inability to demonstrate the microbial metabolism of persistent pesticides and other slowly biodegradable compounds may result from an emphasis upon techniques designed to select for microorganisms that can metabolize such compounds to the extent that the molecule is utilized as the sole source of carbon and energy to support growth of the isolate. The standard method employed since the earliest days of microbiology, namely the elective culture, has been shown to have its drawbacks (1), the most critical of which is that many bacteria are capable of metabolizing substrates but cannot utilize such substrates as sole sources of energy or of carbon for proliferation. The relevance of this phenomenon, termed "cometabolism" (6), to natural ecosystems has recently been considered by Horvath and Alexander (5).

The microbial degradation of DDT illustrates the phenomenon of cometabolism quite well. All attempts to isolate microorganisms capable of utilizing this insecticide as a sole carbon source for growth have proved futile, although DDT is known to be transformed by bacteria. Because the organisms isolated to date are apparently incapable of effecting cleavage of either of the benzene rings of the molecule, only the removal of one of the carbons from DDT has been demonstrated (10, 11). In the present study, the elective culture technique has been employed to obtain a microorganism capable of growing on diphenylmethane, a nonchlorinated analogue of DDT, and an investigation was undertaken to show cleavage of both rings of diphenyl-

methane. The similarity in the pathways by which diphenylmethane and products generated from DDT are degraded, the latter by cometabolism, is the subject of another report (Focht and Alexander, *in preparation*).

MATERIALS AND METHODS

Isolation and growth. A bacterium capable of utilizing diphenylmethane as its sole carbon source was obtained from sewage effluent by the enrichment technique with diphenylmethane as the sole organic constituent in the solution. The isolated culture was grown in a solution containing 0.5% diphenylmethane, 10 mmoles of K_2HPO_4, 3 mmoles of NaH_2PO_4, 10 mmoles of $(NH_4)_2SO_4$, 1 mmole of $MgSO_4$, 0.5 mg of $FeSO_4$, and 0.5 mg of $Ca(NO_3)_2$ per liter of distilled water; the final *p*H was 7.2. To determine whether the isolate used the test compounds as sole carbon sources for growth, the substance was included in the medium at a concentration of 0.1%, and catechol, 3- and 4-methylcatechols, and the hydroxydiphenylmethanes were included at concentrations of 0.1 and 0.04%, respectively. Growth was carried out at room temperature.

Manometry. Resting cell suspensions were prepared from cultures grown in 500-ml baffled flasks containing 200 ml of the salts solution supplemented with the desired carbon source. The bacteria were harvested during the stationary phase, at 36 hr, by centrifugation, and they were washed and resuspended three times in 0.05 M phosphate buffer (*p*H 7.2). Centrifugation of diphenylmethane-grown cells was carried out at room temperature to prevent precipitation of diphenylmethane (melting point, 22 C). A 10-fold dilution of the cell suspension used for manometric determinations had an optical density (OD) in an 18-mm test tube of 0.40 at 540 nm.

Oxygen uptake was determined manometrically at 30 C (9). The side arm received 1.26 *μ*moles of di-

phenylmethane or 1.0 μmole of the other substrates, and 2.0 ml of the cell suspension was added to the main compartment. The final liquid volume was 3.2 ml. Nonliquid water-insoluble substrates were dissolved in acetone and introduced into the main compartment. The acetone was evaporated off, and 2.0 ml of buffer was added; in these instances, the cell suspension (OD 8.0 at 660 nm) was placed in the side arm. The gas exchange was corrected for endogenous respiration.

Cell-free extracts were prepared from cultures grown in 2 liters of a diphenylmethane-inorganic salts medium. The cells were collected by centrifugation, washed as previously described, suspended to give a thin paste, and disrupted with a Heat Systems-Ultrasonics probe for 4 min in an ice-cooled bath. The extract was usually freed from cells by centrifugation at 27,000 × g for 30 min, and it was stored under N_2 at −10 C. Sufficient extract was added to each Warburg flask to give 10 mg of protein, as determined by the biuret method (2), and 2.0 μmoles of substrate was used.

Extraction procedure. The supernatant from a culture grown in 1.0 liter of diphenylmethane-salts medium was extracted with three 100-ml portions of hexane. The solvent layer was discarded, and the aqueous layer was acidified with 5.0 ml of concentrated H_3PO_4 and extracted with three 200-ml portions of diethyl ether. The ether layer was dried over anhydrous Na_2SO_4 and extracted with three 50-ml portions of 0.1 M Na_2CO_3. The Na_2CO_3 phase was acidified with 5 ml of H_3PO_4 and extracted with three 30-ml portions of ether. The ether portions were pooled and the solvent was evaporated.

Analytical techniques. For thin-layer chromatography, the residue from the extracted culture supernatant was taken up in a minimal amount of ether and spotted across the width of a Silica Gel HFr₂₅₄ plate (20 by 20 cm; E. Merck AG, Darmstadt). The plate was developed to a height of 16 cm in a benzene-dioxane-acetic acid (10:3:1, v/v) solvent system. After location of the bands by means of an ultraviolet lamp, the region of interest was scraped off the plate, the compound was eluted with ether, and the resulting solution was filtered and evaporated to dryness.

Melting points were determined with a Nagle hot plate microscope. Infrared spectra were obtained by using a Beckman double-beam infrared spectrophotometer (model IR 10). Mass spectral analyses were performed with a Perkin-Elmer 270 mass spectrometer equipped with a Honeywell 2106 visicorder. Crystalline samples were introduced through the solids probe inlet. Methyl esters of components in the extracts were prepared by reacting with BCl_3 in methanol. Combination gas-liquid chromatography and mass spectrometry of methyl esters was carried out by using a DC 200 column (2 m by 3 mm) with an inlet to the mass spectrometer at a flow rate of 25 ml/min at 180 C.

RESULTS

The isolate was identified as a strain of *Hydrogenomonas* on the basis of its morphological and

TABLE 1. *Utilization of aromatic compounds by resting cell suspensions of diphenylmethane-grown Hydrogenomonas sp.*

Substrate	Growth	Oxygen consumed per micromole of substrate	Lag period
		μmole	*min*
Diphenylmethane	+	12.3	0
Benzhydrol	+	10.5	0
Benzophenone	−	5.8	0
o-Hydroxydiphenylmethane	−	3.3	0
p-Hydroxydiphenylmethane	−	3.2	0
Phenylacetic acid	+	4.7	0
Homogentisic acid	+	3.0	0
Phenylglyoxylic acid	+	3.1	45
Benzoic acid	+	4.0	15
Gentisic acid	+	3.1	40
2,3-Dihydroxybenzoic acid	−		
Protocatechuic acid	+	2.8	20
Catechol	−	2.1	0
3-Methylcatechol	−	2.0	0
4-Methylcatechol	−	2.0	0

biochemical characteristics (3, 8). It is a gram-negative, polar flagellated rod which hydrolyzes gelatin, gives positive reactions for catalase and cytochrome oxidase, and is negative in indole production, the methyl red test, and nitrate reduction. It grows autotrophically in an atmosphere of H_2, CO_2, and O_2. Development is rapid in the diphenylmethane-salts medium, and the maximum cell density is achieved in 36 hr with a 0.5% inoculum.

The results of a study to determine the capacity of the bacterium to oxidize and to grow at the expense of diphenylmethane, structurally related compounds, and possible degradation products are summarized in Table 1. Resting cell suspensions were prepared from diphenylmethane-grown cultures. Eight of the chemicals supported growth, whereas all but 2,3-dihydroxybenzoic acid were oxidized by resting cells. Several compounds structurally related to diphenylmethane, such as benzhydrol and benzophenone, could thus be metabolized, although the extent of their oxidation was less than that of diphenylmethane (Fig. 1).

Of all of the compounds listed in Table 1, cell-free extracts oxidized only catechol, 3- and 4-methylcatechols, and homogentisic acid. Ring fission of the three catechols by the extracts, the likely consequence of the oxidation, resulted in the uptake of 1 mole of O_2 per mole of substrate. Bright yellow products appeared in the incuba-
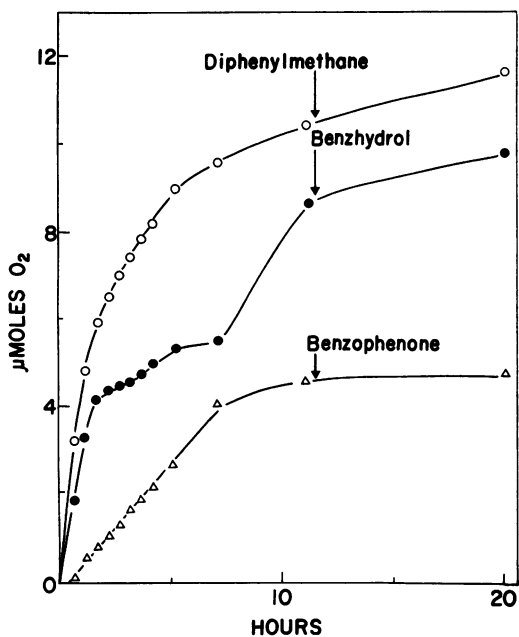
FIG. 1. *Oxidation of diphenylmethane, benzhydrol, and benzophenone by washed cells grown on diphenylmethane.*
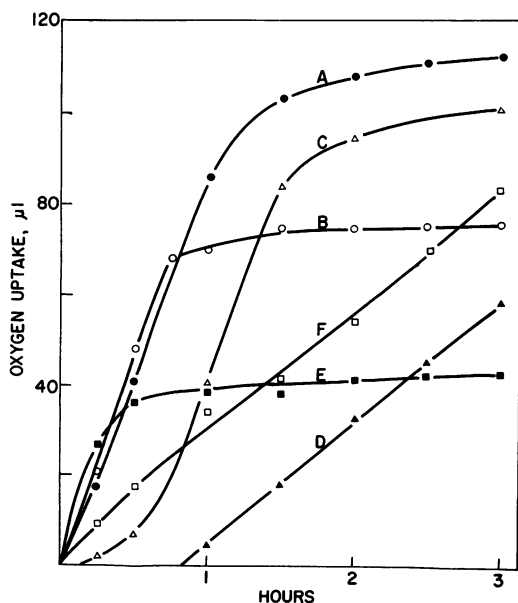

FIG. 2. *Oxidation of phenylacetic acid (A), homogentisic acid (B), benzoic acid (C), phenylglyoxylic acid (D), catechol (E), and diphenylmethane (F) by washed cells grown on phenylacetic acid.*
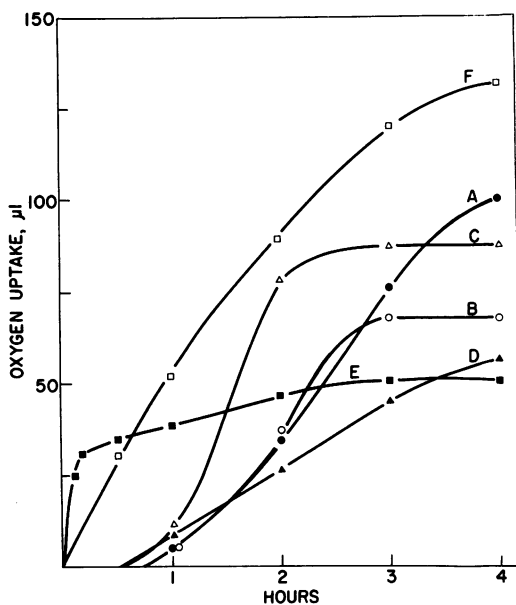

FIG. 3. *Oxidation of phenylacetic acid (A), homogentisic acid (B), benzoic acid (C), phenylglyoxylic acid (D), catechol (E), and diphenylmethane (F) by glucose-grown cells.*

tion mixture, but the colors were abolished when the liquid was acidified. The enzyme system catalyzing this reaction was highly oxygen-labile. These characteristics are those of a metapyrocatechase (4, 7). Homogentisic acid was likewise oxidized with the uptake of 1 mole of O_2 per mole of substrate within a 10-min period, although no yellow-colored product was observed.

Cells grown on phenylacetic acid readily oxidized phenylacetic acid, homogentisic acid, catechol, and diphenylmethane (Fig. 2). Benzoic acid and phenylglyoxylic acid were both oxidized but only after a lag period. Cells grown on 0.5% glucose oxidized diphenylmethane and catechol without an initial lag period. Phenylacetic, homogentisic, phenylglyoxylic, and benzoic acids, however, were oxidized after a lag period (Fig. 3). Extracts prepared from diphenylmethane-grown cultures by centrifugation of the ruptured cells at 5,000 × g showed no O_2 uptake when initially incubated with benzoic or phenylglyoxylic acid, but, interestingly, some activity was observed after a lag phase.

A compound was isolated from extracts of the culture supernatant, and it was separated by thin-layer chromatography. It was identified as phenylacid by its melting point (77 C) and infrared and mass spectra, all of which were identical to the characteristics of the authentic compound.

Phenylglyoxylic and benzoic acids were found in the culture fluid in small amounts, and they were identified as the methyl esters on the basis of their retention times in the gas chromatograph (5:00 and 3:25 min) and by means of mass spectrometry. The retention times and mass spectra of the methyl esters of phenylglyoxylic and benzoic acids were identical to those of the authentic methyl esters.

DISCUSSION

The initial attack upon diphenylmethane does not involve an oxidation of the methylene carbon. This view is supported by (i) the biphasic pattern of benzhydrol oxidation, presumably the result of a de novo synthesis of enzymes not functioning in diphenylmethane degradation; (ii) the incomplete oxidation of benzophenone; and (iii) the unsuitability of benzophenone as a growth substrate (although it is metabolized, by contrast with diphenylmethane). If indeed there is no oxidation of the methylene carbon before ring cleavage, the first step in metabolism would appear to be different from that which seemingly occurs in the conversion by *Aerobacter aerogenes* of p,p'-dichlorodiphenylmethane (DCM) to the corresponding benzhydrol and benzophenone (11). The role of a benzyl phenol, like o- or p-hydroxydiphenylmethane, as an intermediate in diphenylmethane degradation is unlikely inasmuch as such compounds, although oxidized, fail to support growth. They are probably metabolized by reactions different from those involved in diphenylmethane degradation.

The demonstration that phenylacetic acid is generated in diphenylmethane degradation provides definitive evidence that there is no oxidation of the methylene carbon. Since phenylglyoxylic and benzoic acids were present in only trace amounts, it is possible that they are rapidly metabolized and hence fail to accumulate. On the other hand, the data show that intact cells oxidized phenylglyoxylic acid far more slowly than phenylacetic acid and that both benzoic and phenylglyoxylic acids were utilized but only after a lag period. These observations make the role of the latter two compounds in the pathway of diphenylmethane decomposition somewhat ambiguous.

The extent of the oxidation of phenylacetic acid shows that its ring is cleaved; hence, both rings of the original diphenylmethane can be broken by *Hydrogenomonas* sp. Phenylacetic acid may not be metabolized via phenylglyoxylic or benzoic acids but rather via homogentisic acid.

This view is supported by the immediate oxidation of homogentisic acid by cell-free extracts and by suspensions of cells grown on phenylacetic acid or diphenylmethane. Moreover, inasmuch as the enzymes catalyzing the degradation of phenylacetic acid are inducible whereas those effecting initial steps in the degradation of diphenylmethane are constitutive, the oxidation of the two benzene rings of the latter compound must be brought about by different enzyme systems.

The findings reported herein take on especial relevance in regard to pesticide metabolism when the results are compared with those obtained in the cometabolism of DCM, a metabolite produced in the biodegradation of DDT. Thus, *Hydrogenomonas* sp. acts upon DCM and forms p-chlorophenylacetic acid (D. D. Focht et al., Bacteriol. Proc., p. 8–9, 1970), presumably by a pathway analogous to that considered herein. Further studies are necessary to establish how the two benzene rings are attacked and the ultimate fate of the fragments.

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