Incorporation of Oxygen-18 into Benzene by *Pseudomonas putida**

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ABSTRACT: Appropriately induced cultures of *P. putida* oxidize toluene and benzene at approximately equal rates. A mutant strain of this organism has been designated *P. putida* 39/D.

When *P. putida* 39/D is grown on glucose, in the presence of benzene, *cis*-1,2-dihydroxycyclohexa-3,5-diene accumulates in the culture medium. The microbiologically produced product is identical with a synthetic sample of *cis*-1,2-dihydroxycyclohexa-3,5-diene. Experiments with isotopic oxygen shows that two atoms of atmospheric oxygen are incorporated into *cis*-1,2-dihydroxycyclohexa-3,5-diene. These results indicate that enzymatic oxidation of benzene by *P. putida* is different to reported mechanisms for the microsomal oxidation of benzene.

In a recent communication (Gibson *et al.*, 1968b) we proposed the reaction sequence shown in Figure 1 for the enzymatic formation of catechol from benzene. The implication of *cis*-1,2-dihydroxycyclohexa-3,5-diene (*cis*-1,2-dihydro-1,2-dihydroxybenzene) as an intermediate in the degradation of benzene is at variance with reported mechanisms for the enzymatic degradation of aromatic hydrocarbons. Available evidence indicates that, in both microbial and mammalian systems, *trans*-dihydrodiols are formed from different aromatic substrates (Walker and Wiltshire, 1953; Griffiths and Evans, 1965; Booth and Boyland, 1949; Young, 1947; Smith *et al.*, 1950; Sato *et al.*, 1963; Jerina *et al.*, 1968a). The *trans*-diols are presumably formed by the hydrolysis of expoxide precursor molecules (Boyland and Booth, 1962; Holtzman *et al.*, 1967).

Recently Jerina *et al.* (1968a) have shown that 1,2-benzene oxide is converted by "epoxide hydrase" into *trans*-1,2-dihydro-1,2-dihydroxybenzene. The enzyme is found in both the microsomal and soluble fractions of rabbit liver and converts different epoxides into 1,2-diols. Rabbit liver microsomes also oxidize naphthalene to 1,2-naphthalene oxide which can then be enzymatically converted into *trans*-1,2-dihydro-1,2-dihydroxynaphthalene (Jerina *et al.*, 1968b).

We now wish to report the accumulation of *cis*-1,2-dihydro-1,2-dihydroxybenzene by a mutant strain of *P. putida*. Experiments with isotopic oxygen reveal that two atoms of oxygenare incorporated into the aromatic nucleus. These observations support the reaction sequence shown in Figure 1 and suggest that the involvement of 1,2-benzene oxide in the microbial degradation of benzene is unlikely.

Materials and Methods[1]

Organism and Growth Conditions. P. putida (wild type) and *P. putida* 39/D were isolated and grown as described previously (Gibson *et al.*, 1968b; Gibson *et al.*, 1970). Considerable difficulty was encountered in experiments designed to show the formation of *cis*-1,2-dihydro-1,2-dihydroxybenzene from benzene. Toluene allows growth when it is supplied in the vapor phase to liquid cultures. Benzene under the same conditions inhibits the growth of *P. putida* (wild type). The addition of benzene directly to the culture medium in varying concentrations (0.1–1.0 g/l.) did not result in growth of *P. putida* (wild type). The addition of (1/4 × 1 in.) sealed dialysis sacs, containing 1.0 ml of benzene, to the culture medium allowed good growth of *P. putida* (wild type). However the results were not always reproducible and it was assumed that this was due to variations in the pore size of the dialysis tubing. Reproducible results were obtained by autoclaving 10 ml of paraffin wax in a 500-ml flask. To the molten wax 1.0 ml of benzene was added and the wax was allowed to solidify. At this time 200 ml of sterile mineral salts medium (Stanier *et al.*, 1966) was added aseptically to the flask containing the paraffin–benzene mixture. This medium, when inoculated with *P. putida* (wild type) and shaken on a reciprocal shaker at 30°, allowed good growth of the organism. This procedure provides a reliable method for investigating the growth of microorganisms on benzene. *P. putida* showed no growth when benzene was omitted from the paraffin. No contamination occurred when the mineral salts solution was replaced with 200 ml of uninoculated (5%, w/v) yeast extract.

A modification of the above procedure was used for the $^{18}O_2$ experiment. Benzene was added to molten paraffin wax to a final concentration of 10% and 1.5-ml portions were poured into a sterile, chilled spotting tile. After solidification of the wax two pellets were transferred to 100 ml of cell suspension in a 500-ml flask.

Cultures of *P. putida* 39/D were grown on solid medium in large Petri dishes (diameter 20 cm; height 2 cm). Mineral salts medium supplemented with 0.4% glucose and 2% agar (Bacto-Agar, Difco certified) was used to support growth. The inoculum for these plates was obtained from *P. putida* 39/D which had been grown for 18 hr on yeast extract–nutrient agar slants. The amount of inoculum for each plate was 0.2 ml of a cell suspension which gave a reading of 150 Klett Units (660 mμ, red filter, 1:10 dilution). The benzene

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[1] Abbreviations used are as listed in *Biochemistry 5*, 1445 (1966).

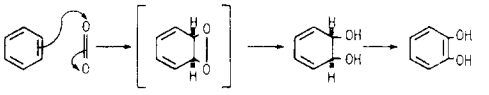

FIGURE 1: Proposed reaction sequence for the enzymatic formation of catechol from benzene.

oxygenase system was induced by incubating each plate in the presence of low concentrations of benzene. This was achieved by supplying the benzene in small (4 × 70 mm) cotton-plugged glass tubes. Each tube was placed in the center of the Petri dish lid and the cells were allowed to grow for 15 hr. At this time the cells were fully induced to convert benzene into *cis*-1,2-dihydro-1,2-dihydroxybenzene. The cells were washed five times with a total volume of 1200 ml of 0.067 M phosphate buffer, pH 7.5. Finally the cells were suspended in the same buffer to give a standardized cell suspension with a reading of 170 Klett units (660 mμ, red filter).

Assay of cis-1,2-Dihydro-1,2-dihydroxybenzene. Samples (1.0 ml) were taken from growing cultures or washed cell suspensions and centrifuged at 10,000*g* for 5 min. From the clear supernatant solution 0.05 ml was carefully transferred to a silica cuvet and diluted to 3.0 ml with distilled water. Readings were taken at 262 mμ against a control sample prepared from a culture containing all components except benzene. The concentration of *cis*-1,2-dihydro-1,2-dihydroxybenzene was calculated using $\epsilon_{262\ m\mu} = 3715$ (Nakajima *et al.*, 1959).

Incorporation of ^{18}O into Benzene. The standardized cell suspension (100 ml) was placed in a 500-ml Büchner flask. The flask was modified to contain two paraffin–benzene pellets in a separate chamber. The chamber (volume 8.0 ml) was obtained by attaching a length of rubber tubing (diameter, 30 mm; length, 50 mm) to the neck of the flask. The paraffin–benzene pellets were prevented from coming into contact with the standardized cell suspension by means of a screw clip (Figure 2). The flask was alternatively evacuated and flushed with pure nitrogen. After the fourth evacuation 100 ml of isotopic oxygen was introduced into the flask. The flask was then brought to atmospheric pressure with nitrogen. The final atmosphere contained approximately 81.1% N_2, 18.3% $^{18}O_2$, and 0.6% $^{16}O_2$. The paraffin–benzene pellets were dropped into the cell suspension by opening the clamp at the neck of the flask. The reaction flask was incubated at 27° for 30 hr on a reciprocal shaker. At this time the cells were removed by centrifugation and the clear supernatant solution (pH 7.5) evaporated to dryness at room temperature. The residue was extracted with methanol and the solvent was removed to leave 67 mg of pale yellow solid. Three recrystallizations from petroleum ether (60–80°) gave 15 mg of pure *cis*-1,2-dihydro-1,2-dihydroxybenzene.

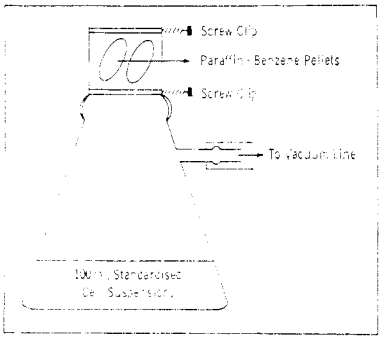

FIGURE 2: Apparatus used for investigating the enzymatic incorporation of $^{18}O_2$ into benzene.

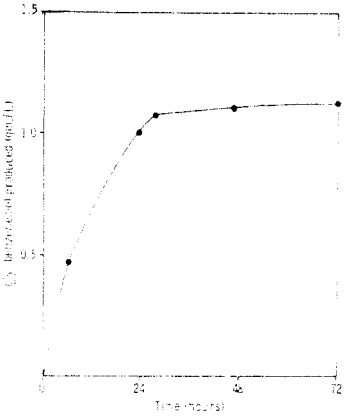

FIGURE 3: The production of *cis*-1,2-dihydro-1,2-dihydroxybenzene from benzene by induced cells of *P. putida* 39/D. Growth conditions and assay techniques are described in the text.

Reagents. Pure refined paraffin (Texwax) was from Texaco, Inc. Spectral Grade benzene was from J. T. Baker Chemical Co., Phillipsburg, N. J. $^{18}O_2$ (93.48%) was obtained from Volk Radiochemical Co., Burbank, Calif. All other chemicals were from sources described previously (Gibson *et al.*, 1968a,b).

Analytical Methods. All procedures have been described previously (Gibson *et al.*, 1970).

TABLE 1: Substrate Specificity of Washed Cell Suspensions of *P. putida* (Wild Type) and *P. putida* 39/D.[a]

Substrates	μl of O_2 Consumed/10 min	
	Wild Type	39/D
Benzene	76	18
Toluene	81	15
cis-1,2-Dihydro-1,2-dihydroxybenzene	79	0
cis-2,3-Dihydro-2,3-dihydroxytoluene	76	0
trans-1,2-Dihydro-1,2-dihydroxybenzene	8	2
Catechol	75	63
3-Methylcatechol	74	54

[a] Warburg flasks contained, in a final volume of 3 ml: KH_2PO_4 buffer, 160 μmoles; cell suspension, 1.0 ml; and substrate 5 μmoles in 0.2 ml of *N*,*N*-dimethylformamide. Results are corrected for endogenous respiration in the absence of substrate.

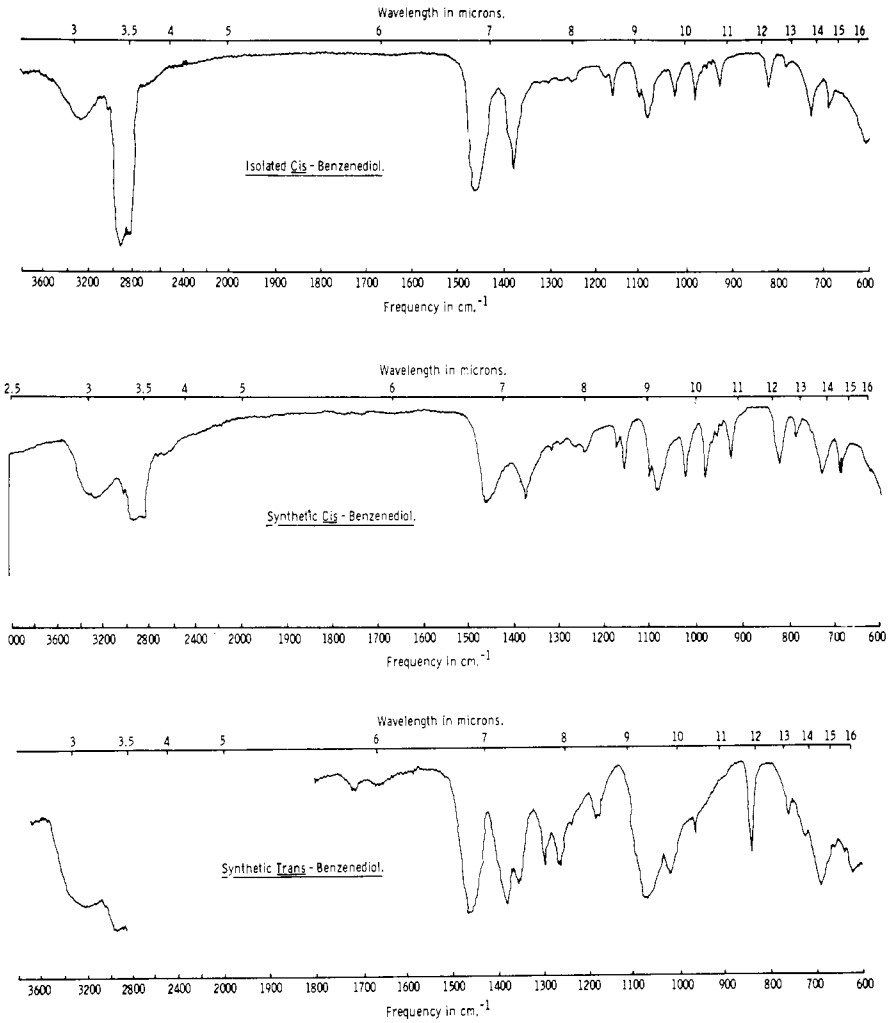

FIGURE 4: Infrared spectra of isolated and synthetic *cis*-1,2-dihydro-1,2-dihydroxybenzene and synthetic *trans*-1,2-dihydro-1,2-dihydroxybenzene. Samples were mulled in Nujol and their spectra were recorded on a Beckman spectrophotometer, Model 1R7.

Results

Cells of *P. putida* (wild type), after growth on benzene, oxidized benzene, toluene, *cis*-1,2-dihydro-1,2-dihydroxybenzene, *cis*-2,3-dihydro-2,3-dihydroxytoluene, catechol, and 3-methylcatechol at approximately equal rates (Table I). In contrast benzene-induced cells of *P. putida* 39/D oxidized benzene and toluene at approximately 25% of the rate observed with the wild-type organism. The mutant strain showed a much greater activity with catechol and 3-methylcatechol, oxidizing these compounds at approximately 70–80% of the rate observed with *P. putida* (wild type). The *trans* isomer of 1,2-dihydro-1,2-dihydroxybenzene was not oxidized by either organism. At the end of the oxidation experiment the contents from each Warburg flask were extracted with ethyl acetate. Chromatography of each organic extract in chloroform–acetone (80:20) revealed that the only detectable transformation products were *cis*-1,2-dihydro-1,2-dihydroxybenzene and *cis*-2,3-dihydro-2,3-dihydroxytoluene. These compounds were produced from benzene and toluene, respectively, by *P. putida* 39/D.

The rate of production of *cis*-1,2-dihydro-1,2-dihydroxybenzene by *P. putida* 39/D is shown in Figure 3. In this experiment the organism was grown under the same conditions as those described for investigating the fixation of $^{18}O_2$ (see Methods). After 30 hr the cells were removed by centrifuging at 10,000*g* for 15 min. The supernatant solution was evaporated to dryness at room temperature and the residue was dissolved in methanol. The methanol extract was filtered and the solvent was removed to leave 78 mg of semicrystalline residue. Three crystallizations from petroleum ether (60–80°) gave 23 mg of pure *cis*-1,2-dihydro-1,2-dihydroxybenzene. A mixture melting point showed no depression. The infrared spectra of the isolated 1,2-dihydro-1,2-dihydroxybenzene and synthetic *cis*- and *trans*-1,2-dihydro-1,2-dihydroxybenzene are shown in Figure 4. These results show that the enzymatically produced compound is *cis*-1,2-dihydro-1,2-dihydroxybenzene.

The above experiment was repeated in the presence of $^{18}O_2$. The crystalline product was identified as *cis*-1,2-dihydro-1,2-dihydroxybenzene by mixture melting point with synthetic *cis*-1,2-dihydro-1,2-dihydroxybenzene. The infrared spectrum of the isolated compound was completely superimposable on the infrared spectrum given by synthetic *cis*-

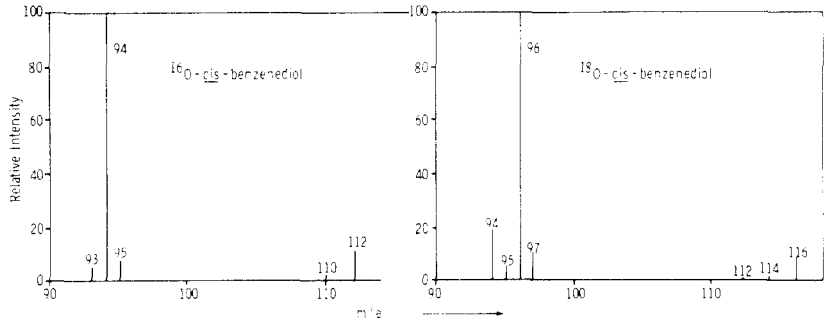

FIGURE 5: Mass spectra of ^{16}O *cis*-1,2-dihydro-1,2-dihydroxybenzene (left) and ^{18}O *cis*-1,2-dihydro-1,2-dihydroxybenzene (right). Spectra were recorded on a Consolidated Engineering Company 21102, modified mass spectrometer with an ionizing current of 50 μA. The temperature of the inlet reservoir was 115°

1,2-dihydro-1,2-dihydroxybenzene. The mass spectra of *cis*-1,2-dihydro-1,2-dihydroxybenzene formed in the presence of $^{16}O_2$ and also $^{18}O_2$ are shown in Figure 5. The compound formed from benzene in the presence of $^{16}O_2$ shows a base peak at *m/e* 94 (phenol ion), a parent peak at *m/e* 112, and a smaller (p−2) peak at *m/e* 110. This spectrum is identical with the spectrum given by synthetic *cis*-1,2-dihydro-1,2-dihydroxybenzene. The compound produced in the presence of $^{18}O_2$ shows a base peak at *m/e* 96 and smaller peaks at *m/e* 94, 95, and 97. The parent peak at *m/e* 116 clearly indicates the incorporation of two atoms of oxygen into *cis*-1,2-dihydro-1,2-dihydroxybenzene. The small peak at *m/e* 112 is in accord with the formation of *cis*-1,2-dihydro-1,2-dihydroxybenzene from the low percentage of $^{16}O_2$ in the medium. However the peak at *m/e* 114 is not easily explained. To see if *cis*-1,2-dihydro-1,2-dihydroxybenzene exchanged its ^{18}O with the medium the isolated compound was incubated with sterile culture medium for 10 days. At this time *cis*-1,2-dihydro-1,2-dihydroxybenzene was reisolated and examined by mass spectrometry. The small peak at *m/e* 114 was still present and its ratio to the parent peak at *m/e* 116 was unaffected.

Discussion

This communication describes the isolation of *cis*-1,2-dihydro-1,2-dihydroxybenzene, an intermediate compound in the microbial degradation of benzene.

The substrate specificity of benzene-grown cells is analogous to that reported for toluene-grown cells of the same organism (Gibson *et al.*, 1968b). Since benzene is not an intermediate in toluene oxidation and *vice versa*, it appears that the enzymes induced by these substrates are not very specific.

The incorporation of two atoms of oxygen, presumably from the same molecule, into *cis*-1,2-dihydro-1,2-dihydroxybenzene suggests that a cyclic peroxide intermediate (Figure 1) may be involved in benzene oxidation. Analogous intermediate compounds have been postulated in the oxidation of anthranilic acid to catechol (Kobayashi *et al.*, 1964) and the oxidation of 2-fluorobenzoic acid to catechol (Milne *et al.*, 1968). It appears unlikely, although the possibility cannot be discounted, that 1,2-benzene oxide is a precursor of *cis*-1,2-dihydro-1,2-dihydroxybenzene. Such a reaction would involve the stereospecific oxidative opening of an epoxide to give a *cis*-dihydrodiol. The small peak at *m/e* 114 in the mass spectrum of isotopically labeled *cis*-1,2-dihydro-1,2-dihydroxybenzene, is indicative of an epoxide intermediate which undergoes hydration to form *trans*-1,2-dihydro-1,2-dihydroxybenzene. However the infrared spectrum of the isolated product did not give any indication of the presence of the *trans* isomer. It seems more likely that the peak at *m/e* 114 is a p−2 peak. However another explanation is possible. Since the isotopically labeled *cis*-1,2-dihydro-1,2-dihydroxybenzene did not undergo isotopic exchange under the conditions of the experiment it is conceivable that an intermediate compound which can exchange ^{18}O may be involved in the formation of *cis*-1,2-dihydro-1,2-dihydroxybenzene. It is well known that benzoquinone exchanges completely with water (Fesenko and Gragerov, 1955) and the possibility that *o*-benzoquionone may be a transient intermediate in the conversion of benzene into *cis*-1,2-dihydro-1,2-dihydroxybenzene is at present being investigated.

Acknowledgments

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