Oxidative Degradation of Aromatic Hydrocarbons by Microorganisms. II. Metabolism of Halogenated Aromatic Hydrocarbons*

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ABSTRACT: *Pseudomonas putida*, grown with toluene as sole source of carbon, oxidized chloro-, bromo-, iodo-, and fluorobenzenes to their respective 3-halogenated catechol derivatives. The dihydroxy compounds from the first three substrates above were identified by isolation and comparison with synthetic compounds. The investigation of the metabolism of *p*-chlorotoluene resulted in the isolation of two compounds which have been identified as (+)-*cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene and 4-chloro-2,3-dihydroxy-1-methylbenzene.

The enzymatic dehydrogenation of the former compound into the latter catechol has been demonstrated. The role of *cis*-glycols in the microbial metabolism of aromatic compounds is discussed with references to known pathways in mammals.

Our continued interest in the mechanism of oxygen fixation into the aromatic nucleus led us to try to isolate the early intermediates in the degradation of several aromatic compounds. Since both toluene and benzene are metabolized at an extremely rapid rate by *Pseudomonas putida* (Gibson *et al.*, 1968), with little accumulation of reaction products, it seemed probable that co-oxidation (Leadbetter and Foster, 1960; Davis and Raymond, 1961) might be of use in our studies. Halogenated aromatic derivatives were chosen as potential partially metabolizable intermediates. The presence of a halogen atom seems to greatly reduce the biodegradability of many aromatic compounds (Alexander and Lustigman, 1966; Ichihara *et al.*, 1962).

This paper describes the isolation and identification of the products formed from chloro-, bromo-, iodo-, and fluorobenzenes and also *p*-chlorotoluene when these substrates are incubated with cells of *P. putida*, grown with toluene as the sole source of carbon.

Materials and Methods[1]

Organism and Growth Conditions. P. putida was grown in media whose composition has been described previously (Gibson *et al.*, 1968). Toluene was introduced to 50-ml cultures by placing 0.5 ml in a glass bulb suspended above the medium. A hole in the glass tube above the glass bulb allowed the toluene vapor to diffuse into the culture flask. The substrate was introduced to 20-l. cultures by blowing air, at the rate of 1 l./min, through a 500-ml flask containing 100 ml of toluene, and into the culture medium. Halogenated aromatic substrates were introduced into the culture medium in an identical manner. Cell extracts were prepared as described previously (Gibson *et al.*, 1968).

Analytical Methods. Oxygen consumption was measured using conventional manometric techniques. A Cary Model 14 recording spectrophotometer was used for spectrophotometric determinations. Routine infrared spectra were obtained with a Perkin-Elmer 137 spectrophotometer. Samples were mulled in Nujol and placed between NaCl disks.

Nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A-60) at 60 Mcycles in deuterated chloroform or deuterated acetone with tetramethylsilane as internal reference. Chemical shifts are reported in τ values (Tiers, 1961). Mass spectra were determined on an Atlas CH4 mass spectrometer equipped with a direct sample injection system and operated at an ionizing voltage of 70 V and and ionizing current of 10–30 μA.

Catechols were determined colorimetrically by the method of Arnow (1937). Calibration curves were prepared for each synthetic 3-halogenated catechol.

Chromatography systems used were chloroform–acetone (80:20), benzene–methanol (95:5), and benzene (100) (all v/v). Compounds were run on thin-layer sheets (Eastman Chromatogram sheets, type K13OR, silica gel with fluorescent indicator). The compounds were located under ultraviolet light and also by spraying with a 2% solution of 2,6-dichloroquinone-4-chloroimine in ethanol. With this reagent, catechols gave red-brown to purple colors, phenols a blue color, while glycols gave faint grey colors.

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

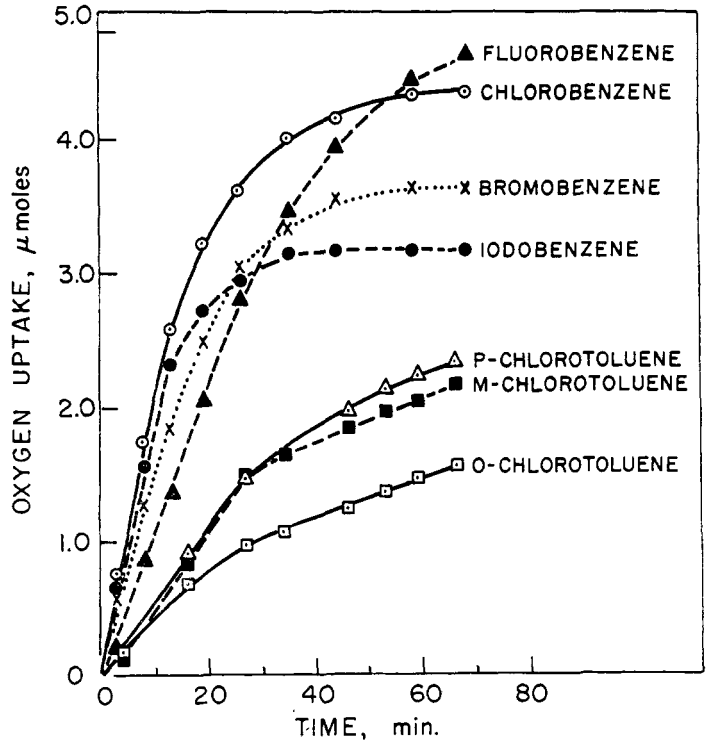

FIGURE 1: Oxidation of halogenated aromatic hydrocarbons by washed cell suspensions of *P. putida*. Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (150 μmoles), cell suspension (1.0 ml), and substrate (5 μmoles) in 0.2 ml of *N,N*-dimethylformamide. Results are corrected for endogenous respiration in the absence of substrate.

Materials. Halogenated salicylaldehydes were synthesized from their respective *o*-halogenated phenols by the Reimer–Tiemann (1876) reaction. The resulting halogenated salicylaldehydes were converted into their respective 3-halogenated catechols by the Dakin (1909) reaction. Although the Reimer–Tiemann reaction gave poor yields (5–10%), the high yields obtained in the subsequent Dakin reaction (70–80%) make this a convenient two-step synthesis for 3-substituted catechols. The *cis* and *trans* isomers of 1,2-dihydroxycyclohexa-3,5-diene were synthesized as described previously (Gibson *et al.*, 1968). All halogenated aromatic hydrocarbons were from Eastman Organic Chemicals and were distilled prior to use. All other chemicals were from sources described previously (Gibson *et al.*, 1968).

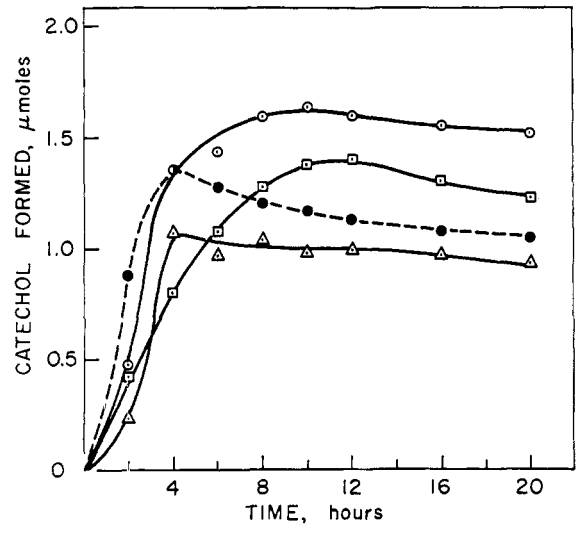

FIGURE 2: Accumulation of halogenated catechols in the culture medium. Conditions as described in the text. (○—○—○) 3-Iodocatechol, (⊡—⊡—⊡) 3-bromocatechol, (●—●—●) 3-chlorocatechol, and (△—△—△) 3-fluorocatechol.

Results

Whole Cell Experiments. Washed cells of *P. putida*, grown with toluene as source of carbon, oxidized chlorobenzene, bromobenzene, and iodobenzene at approximately equal rates. Fluorobenzene was oxidized at half the rate observed with chlorobenzene, while *o*-, *m*-, and *p*-chlorotoluenes were metabolized at a comparatively reduced rate (Figure 1). Chromatographs of the reaction products formed from each of the halogenated aromatic substrates revealed the presence of a catechol derivative. The halogenated benzene derivatives showed the presence of only one reaction product while each of the chlorinated toluene derivatives, in addition to the catechol reaction, revealed the presence of a more polar compound which did not react with ferric chloride. In each case addition of 2 N HCl to the reaction mixtures, prior to chromatography, caused the lower polar compounds to disappear with the concomitant appearance of two phenolic compounds. These observations indicated that the polar compound was probably a dihydrodihydroxy derivative formed from each chlorinated toluene isomer.

Catechol Formation from Halogenated Benzenes. Four cultures of *P. putida* (50 ml) were grown with toluene for 15 hr (see Materials and Methods). At this time the growth substrate in each flask was replaced by one of the following compounds: chlorobenzene, bromobenzene, iodobenzene, or fluorobenzene. Samples (1.0 ml) were taken at intervals and the cells were removed

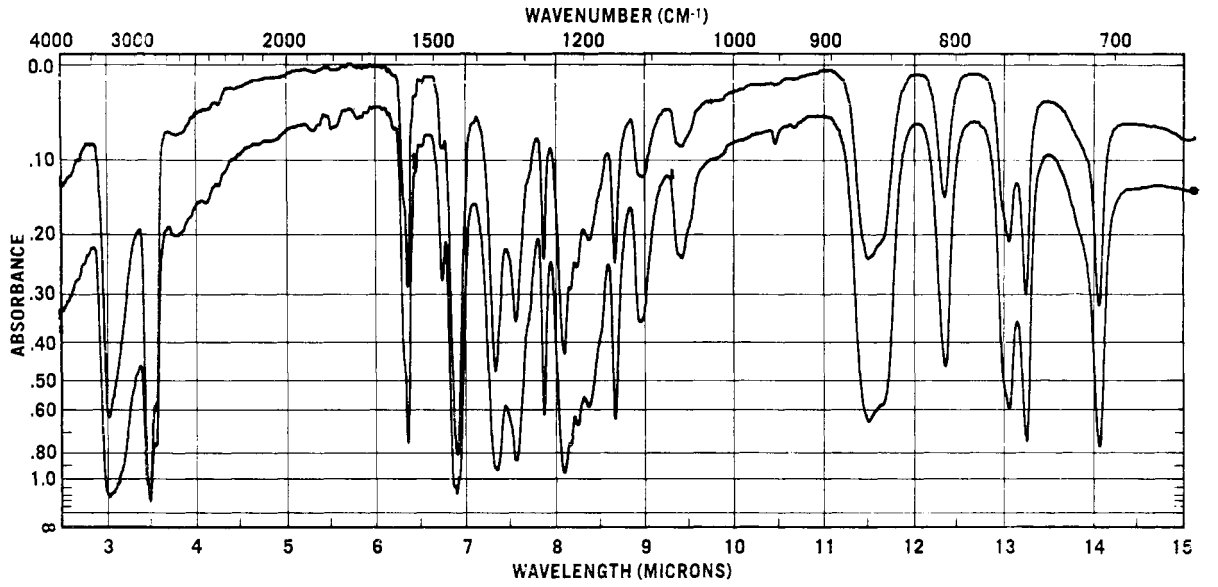

FIGURE 3: Infrared spectra of synthetic (upper curve) and biologically produced (lower curve) 3-indocatechol. Samples were mulled in Nujol and placed between NaCl disks.

by centrifuging at 10,000*g* for 5 min. Each supernatant solution was assayed for catechol by the method of Arnow (1937), and also by following the absorption at λ_{max} which was calculated for each synthetic 3-halogenated catechol. The results from each method agreed to within 10%. After 10 hr the contents of each flask turned brown, and after 24 hr each flask contained a dark brown mixture. It was assumed that the color change was due to autoxidation of the catechols produced from each substrate. The rate of formation of each catechol is shown in Figure 2. These results were used as a guide for later isolation experiments. Due to the conditions used for growth, each substrate was present as a function of its vapor pressure. It is not correct to assume that Figure 2 represents the true affinity of the organism for each substrate. None of the halogenated benzenes would serve as a growth substrate under the conditions described in Materials and Methods.

Isolation of Halogenated Catechols. A 10-l. culture of *P. putida* was grown, with toluene as source of carbon, for 15 hr. At this time, the toluene was replaced with chlorobenzene and incubation was continued for a further 10 hr. The cells were removed by centrifuging and the brown supernatant solution was extracted with 4 l. of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under vacuum at 40°. The brown oily residue (0.8 g) was applied to the top of a silica gel column (40 × 1.5 cm). Elution with chloroform gave 530 mg of a pale yellow oil which was recrystallized from petroleum ether. The crystalline compound (mp 45–47°) gave an infrared spectrum identical with the spectrum given by synthetic 3-chlorocatechol. A mixture melting point showed no depression. A dibenzoyl derivative (mp 108–109°) prepared from the isolated compound gave a melting point identical with that given by a synthetic sample.

In a similar experiment, bromobenzene was substituted for chlorobenzene and resulted in the isolation of 3-bromocatechol (472 mg), mp 39–42°. The isolated compound and synthetic 3-bromocatechol gave identical infrared spectra. A mixture melting point showed no depression.

When iodobenzene was used as substrate, 853 mg of 3-iodocatechol (mp 55–56°) was produced. The enzymatically produced compound gave an infrared spectrum identical with that given by the synthetic compound (Figure 3). A mixture melting point showed no depression.

Fluorobenzene appeared to be toxic under the conditions used for the accumulation of the above halogenated catechols. Extraction of a 10-l. culture filtrate gave 65 mg of a pale yellow oil which would not crystallize. The infrared spectrum of the isolated compound was almost identical with the spectrum given by synthetic 3-fluorocatechol.

Metabolism of p-Chlorotoluene. A 25-l. culture of *P. putida* was incubated with *p*-chlorotoluene for 10 hr. Ethyl acetate extraction of the culture filtrate gave 2.9 g of a brown viscous oil which was dissolved in a small amount of chloroform and applied to the top of a silica gel column (36 × 3 cm). The column was eluted with chloroform and chloroform containing 1% methanol; 15-ml fractions were collected (Table I).

COMPOUNDS I AND II were recognized as phenols by the blue color they gave with 2,6-dichloroquinone-4-chloroimine. The amounts obtained were not sufficient for further characterization.

COMPOUND III. Fractions 20–56 were combined and, after removal of the solvent, the residue was crystallized from petroleum ether: mp 64–65°; λ_{max}^{MeOH} 275 mμ (ϵ_{max} 2862) and 281 mμ (sh) (ϵ_{max} 2769); λ_{max}^{Nujol} 2.85, 3.05, 6.25, 6.32, and 12.85 μ. The nuclear magnetic resonance spectrum showed bands at τ 7.83 (3 H, CH_3 on aromatic ring), 4.63 (2 H, adjacent OH groups, disappears on shaking with D_2O), and 3.22 and 3.42, two perturbed doublets, J = 9.0 cps (2 H, vicinal protons on aromatic ring). The mass spectrum gave an in-

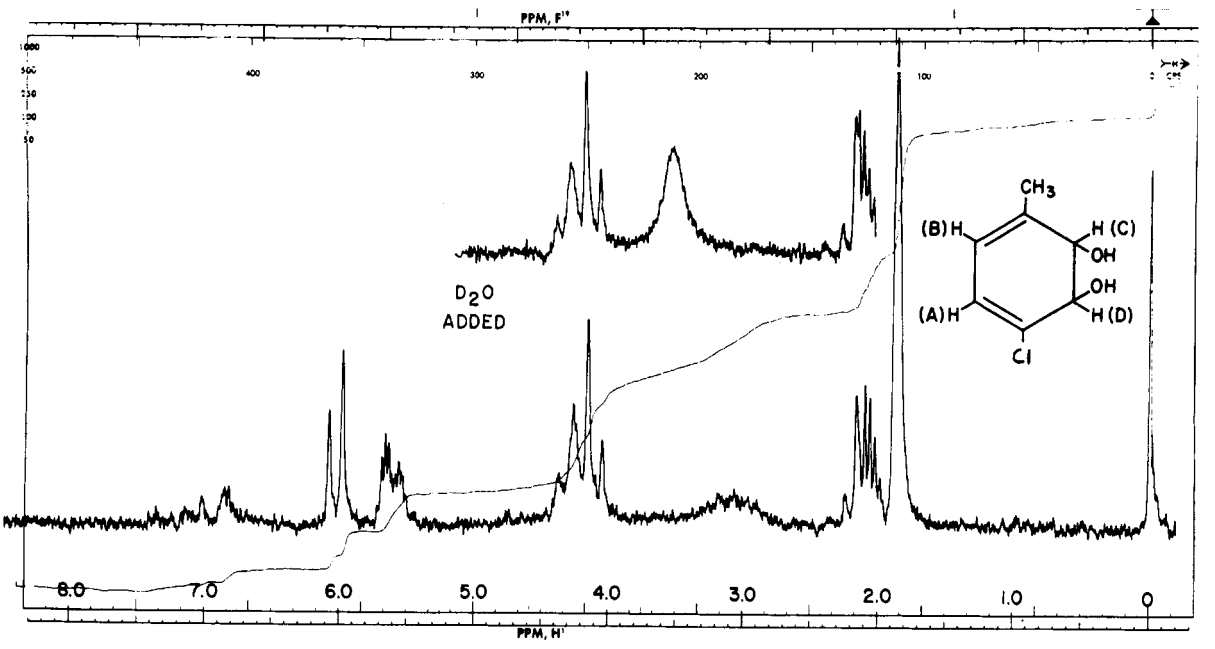

FIGURE 4: Nuclear magnetic resonance spectrum of 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (V) in deuterated acetone.

tense parent peak at *m/e* 158. The P + 2 peak was 33.7% of the parent peak (calcd 32.9%) and indicates the presence of one chlorine atom. The only other intense peak was at *m/e* 125 which corresponds to the loss of chlorine. *Anal.* Calcd for $C_7H_7ClO_2$ (158): C, 53.16; H, 4.43; Cl, 22.15. Found: C, 53.30; H, 4.63; Cl, 22.57. The results establish the structure of compound III as 4-chloro-2,3-dihydroxy-1-methylbenzene.

COMPOUND IV gave a nuclear magnetic resonance spectrum and infrared spectrum identical with those given by 3-methylcatechol. This compound was assumed to have originated from residual growth substrate (toluene) in the culture medium.

COMPOUND V. Fractions 81–115 were combined and the solvent was removed. Crystallization from benzene gave 38 mg of white needles: mp 99–101°; λ_{max}^{water} 276 mμ (ϵ_{max} 6400); λ_{max}^{Nujol} 3.08, 6.06, and 8.98 μ; $[\alpha]_D^{23°}$ +46°. The nuclear magnetic resonance spectrum (Figure 4) was interpreted as a first-order spectrum and showed bands at τ 3.95, H(A) unsymmetrical doublet, J_{AB} = 7.0 cps; τ 4.40, H(B) nine-line multiplet, J_{BA} = 7.0 cps, J_{BCH_3} = 1.5 cps, J_{BC}, finite but small; τ 5.68, H(C), unsymmetrical doublet, J_{CD} = 6.0 cps; τ 5.90, H(D), unsymmetrical doublet, J_{DC} = 6.0 cps; τ 8.17 (3 H, CH_3 on aromatic ring); and τ 6.9 (2 H, adjacent OH groups, disappears on shaking with D_2O). The mass spectrum (Figure 5) showed a prominent parent peak at *m/e* 160. The P + 2 peak was 34.3% of the parent peak and revealed that the compound contained one chlorine atom. Other prominent peaks were seen at *m/e* 142, 125, and 107, corresponding to the loss of H_2O, Cl, and H_2O plus Cl, respectively. The intense peaks at *m/e* 77 (phenyl), 78 (benzene), and 70 (benzene + H) are characteristic of an aromatic ring and are probably formed from the phenol ion which is produced from the parent compound by the elimination of water. *Anal.* Calcd for $C_7H_9ClO_2$ (160); C, 52.43; H, 5.62; Cl, 21.85. Found: C, 52.53; H, 5.83; Cl, 21.67.

These results suggest that V is 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene. Further eivdence for the proposed structure was provided by acid-catalyzed dehydration to a phenol. Compound V (25 mg) was dissolved in 1 N HCl (10 ml) and heated on a steam bath for 10 min. The acid solution was extracted with ethyl acetate and the organic layer was dried over anhydrous sodium sulfate. Removal of the solvent gave 13 mg of an oil which would not crystallize. Chromatography in benzene revealed the presence of a large amount of phenolic compound, R_F 0.25, and a trace amount of a second phenolic compound, R_F 0.43. The oil was dissolved in benzene–petroleum ether (50:50) and applied to the top of a small silica gel column (14 × 1 cm). Elution with the same solvent, followed by crystallization from petroleum ether (bp 30–60°), gave 8 mg of 3-chloro-6-methylphenol (mp 73°) (Hodgson and More, 1926). The other phenol was not obtained in sufficient quantity

TABLE I: Elution Pattern of Products Formed from *p*-Chlorotoluene.[a]

Fractions	Eluent ($CHCl_3$–MeOH)	Wt (mg)	Compd
12–18	100:0	14.5	I + II
20–56	100:0	901	III
57–64	100:0	274	IV
81–115	99:1	49	V

[a] Conditions as described in text.

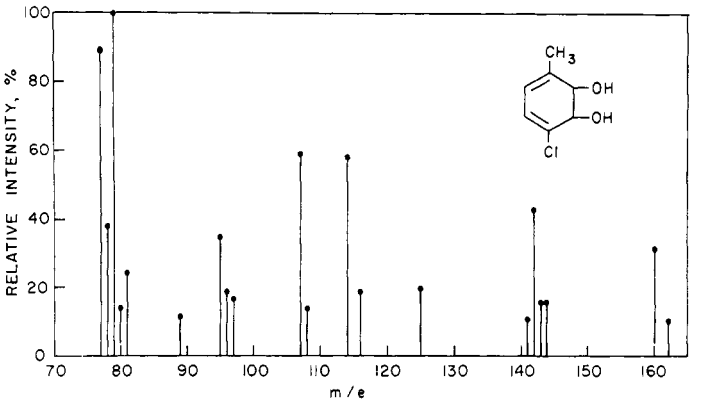

FIGURE 5: Mass spectrum of 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (V).

for characterization but was assumed to be 2-chloro-5-methylphenol. Badger (1949) has shown that when diols of polycylic hydrocarbons are dehydrated, the hydroxyl group which remains on the aromatic nucleus is always located on the carbon atom most activated in electrophilic substitutions. Since a methyl group increases the nucleophilicity of the aromatic ring while a chlorine group has the opposite effect it would be expected that the predominant phenol isomer produced by dehydration of the diol would be 3-chloro-6-methylphenol.

When the isolated diol was incubated with cell extract (prepared from cells grown on toluene) in the presence of NAD^+, dehydrogenation occurred with the formation of 4-chloro-2,3-dihydroxy-l-methylbenzene

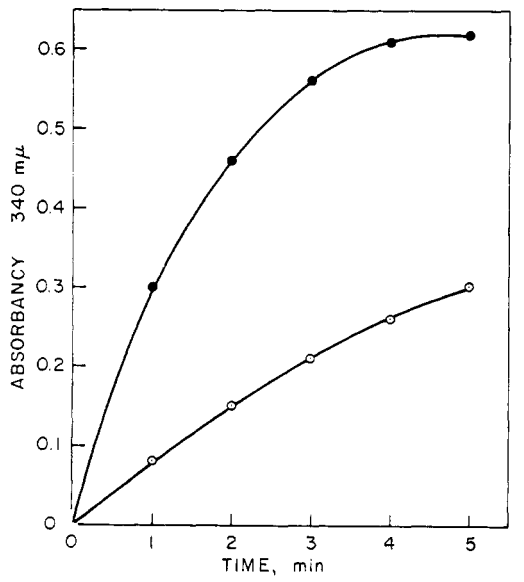

FIGURE 6: Dehydrogenation of 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (V) by cell extract. Anaerobic cuvets contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2) (250 μmoles), NAD^+ (1.0 μmole), V or *cis*-benzene glycol (0.3 μmole), and cell extract (3.2 mg of protein). A reference cuvet contained all components except for the omission of substrate. Cuvettes were made anaerobic by alternately evacuating and flushing with argon for 5 min. The reaction was started by the addition of substrate from the side arm of the cuvet. (●—●—●) *cis*-Benzene glycol and (○—○—○) V.

(Figure 6). The slow dehydrogenation of this compound compared to *cis*-benzene glycol accounts for its appearance in culture filtrates.

Although the above properties establish V as 4-chloro-2,3- dihydroxy - 1 - methylcyclohexa-4,6-diene. they do not indicate whether the compound is the *cis* or the *trans* isomer. In addition, each isomer has two possible conformations (Figure 7). Since the ring is not saturated, the terms aa, ee, and ae refer to pseudodiaxial, pseudodiequatorial, and pseudoaxial–equatorial positions of the hydroxyl groups. As little is known about substituted 1,3-cyclohexadienes, the following procedure was adopted to try to determine whether or not the hydroxyl groups have the *cis* or *trans* configurations.

Potassium Triacetylosmate. Criegee *et al.* (1942), reported that this compound, in glacial acetic acid, forms diesters with *cis*-dihydrodiols with the simultaneous disappearance of the blue color of the reagent. Most *trans*-dihydrodiols do not react. This test has been used to identify *trans*-4-chloro-1,2-dihydroxycyclohexa-4,-6-diene as an intermediate in the metabolism of chlorobenzene by rabbits (Smith *et al.*, 1950). It has also been used to distinguish between *cis*- and *trans*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (Booth and Boyland, 1949). The latter authors have suggested that reaction with potassium triacetylosmic acid is a better diagnostic test for *cis*- and *trans*-glycols than the rate of reaction with lead tetraacetate. However, this reagent must be used with care as it is quite probable that *trans*-glycols whose hydroxyl groups occupy equatorial positions

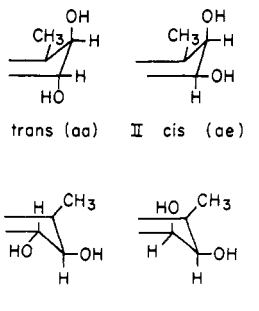

FIGURE 7: Possible conformations of 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (V). For explanation, see text.

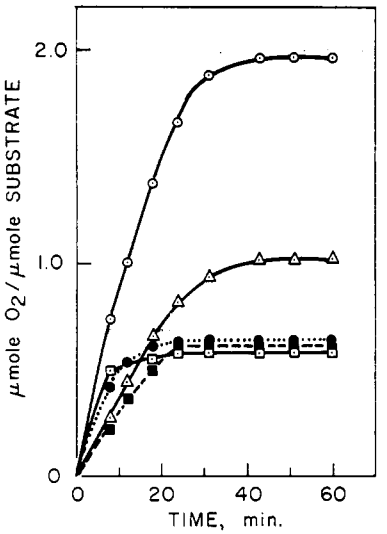

FIGURE 8: Oxidation of halogenated aromatic hydrocarbons by cell extract. Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 (pH 7.2) (160 μmoles), NAD^+ (1.0 μmole), $FeSO_4$ (1.0 μmole), cell extract (12 mg of protein), and substrate (5 μmoles) in 0.2 ml of 25% (v/v) ethanol. Results are corrected for endogenous respiration in the absence of substrate. (○—○—○) Benzene, (△—△—△) fluorobenzene, (●—●—●) bromobenzene, (□—□—□) chlorobenzene, and (■—■—■) *p*-chlorotoluene.

will form diesters with potassium triacetylosmate. Thus Criegee *et al.* (1942) report that *trans*-1,2-dihydroxycyclohexane forms a diester with the reagent. Also the elegant infrared studies of Cole and Jefferies (1956) have shown that in this dihydroxy compound both hydroxyl groups occupy equatorial positions. The reaction of the glycol formed from *p*-chlorotoluene, together with other *cis*- and *trans*-glycols, with potassium triacetylosmate is shown in Table II. The two *cis* compounds and the isolated compound produced an immediate color change in the reagent. None of the *trans*-glycols changed the color of the reagent over a 5-min period. After 30 min, *trans*-1,2-dihydroxycyclohexa-3,5-diene (*trans*-benzene glycol) caused the appearance of a purple color. A similar observation was reported for *trans*-1,2-dihydroxytetrahydronaphthalene (Cook *et al.*, 1950). The results obtained indicate that the isolated glycol is either *cis* (ae) II or IV, or possibly *trans* (ee) III.

Nuclear Magnetic Resonance Spectrum. Karplus (1963) has pointed out the relationship between vicinal coupling constants and the dihedral angle between the protons. Although the relationship is approximate, it has been observed that in six-membered ring systems, coupling constants for diaxial vicinal protons, where the dihedral angle is 180°, lie in the range 8–14 cps, while constants between vicinal axial–equatorial and diequatorial protons are found between 1 and 7 cps (Bhacca and Williams, 1964). The observed coupling constants between the two protons in the isolated glycol (H(C) and H(D); Figure 4) are 6.0 cps and indicate that these protons occupy either the ee or ae positions (I, II, and IV; Figure 7). In view of these observations together with the fact that V reacts with potassium triacetylosmate we propose that the diol formed from *p*-chlorotoluene is (+)-*cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene.

TABLE II: Colorimetric Reaction of *cis*- and *trans*-Diols with Potassium Triacetylosmic Acid.[a]

Compound	Color with PTO[b]
Control (0.5 ml of acetic acid)	Blue
cis-1,2-Dihydroxy-4-cyclohexene	Pale brown
cis-1,2-Dihydroxycyclohexa-3,5-diene	Pale brown
cis-1,2-Dihydroxy-3,4,5,6-tetrachlorocyclohexane	Pale green
?-4-Chloro-2,3-dihydroxy-1-methylcyclohexa-3,5-diene	Pale green
trans-1,2-Dihydroxycyclohexa-4,6-diene	Blue
trans-1,2-Dihydroxy-3,4,5,6-tetrachlorocyclohexane	Blue

[a] Each substrate (0.5 ml) in glacial acetic acid (10 μmoles/ml) was added to 0.5 ml of a 0.001 M solution of potassium triacetylosmate in glacial acetic acid. The color change was recorded after 1 min. [b] PTO, potassium triacetylosmate.

Experiments with Cell Extract. The halogenated benzene derivatives were oxidized at a reduced rate when compared with the oxidation of benzene (Figure 8). Iodobenzene gave essentially the same results as bromobenzene and is not shown in Figure 8. Apart from fluorobenzene, all the other halogenated compounds, or their reaction products, appeared to exert an inhibitory effect on the enzyme system. Only 60% of theoretical oxygen consumption was observed. At the end of the reaction, each reaction mixture which contained a halogenated substrate was deep purple in color. Chromatographic examination of the reaction products revealed that in each case a halogenated catechol had accumulated. When cell extracts were incubated with synthetic 3-chloro-, 3-bromo-, 3-iodo-, and 3-fluorocatechol no oxygen uptake was observed. Similarly, cell extracts failed to metabolize 4-chloro-2,3-dihydroxy-1-methylbenzene which was isolated as a degradation product of *p*-chlorotoluene. The proposed pathway for the metabolism of *p*-chlorotoluene is shown in Figure 9.

Discussion

Hydroxylated compounds have been detected as intermediate compounds in the metabolism of many aromatic substrates. Thus, 3-chlorocatechol was formed when *m*-chlorobenzoate was oxidized by a cell extract prepared from cells grown on benzoic acid (Ichihara *et al.*, 1962). When *m*-fluorobenzoic acid was used as a growth substrate for a *Pseudomonas* sp, 3-fluorocatechol was isolated from the culture filtrate (Goldman *et al.*, 1967). 3-Chlorosalicylic acid and 3-bromosalicylic acid were isolated from the cultures growing on 1-chloro-

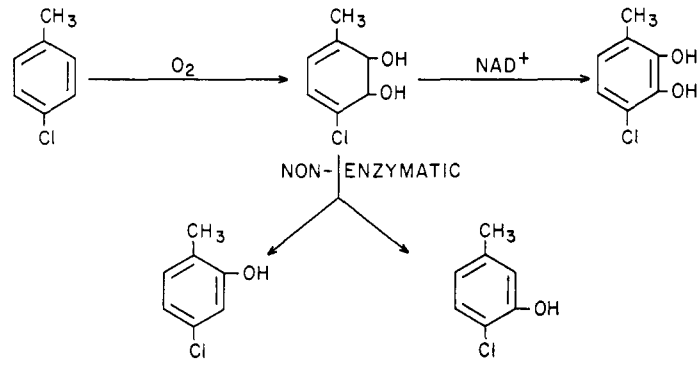

FIGURE 9: Proposed pathway for the metabolism of *p*-chlorotoluene by toluene-grown cells of *P. putida*.

and 1-bromonaphthalenes, respectively (Walker and Wiltshire, 1955). None of the halogenated catechols isolated in the present work were metabolized by either washed cell suspensions or cell extracts of *P. putida*. This is in accord with Alexander's and Lustigman's (1966) observations on the biodegradability of mono- and disubstituted benzenes. These authors found that the presence of a chlorine atom on the benzene ring retarded the rate of biodegradation.

It seems likely from the results of the cell extract experiments that the halogenated catechols inhibit the enzyme system catalyzing the incorporation of oxygen into the aromatic nucleus. They probably chelate the iron which is necessary for the initial oxygenation reaction (Gibson *et al.*, 1968). In this way their inhibitory effect is analogous to the inhibition observed with tiron (catechol-3,5-disulfonic acid) (Gibson *et al.*, 1968). These observations could explain the isolation of relatively low yields of halogenated catechols obtained from chloro-, bromo-, and iodobenzenes, respectively. Fluorobenzene, however, was oxidized with the uptake of 1 mole of oxygen/mole of substrate. The extremely low yield of 3-fluorocatechol in the isolation experiment may be due to the toxic nature of fluorobenzene. It is of interest to note that *P. putida* grows poorly with benzene as substrate and not at all if the water becomes saturated with benzene.

Little is known about the early intermediates involved in the microbial oxidation of mononuclear aromatic hydrocarbons. The implication of *cis*-1,2-dihydroxycyclohexa-3,5-diene (*cis*-benzene glycol) as an intermediate in the enzymatic formation of benzene (Gibson *et al.*, 1968) and the identification of (+)-*cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene as a metabolite in the conversion of *p*-chlorotoluene into 4-chloro-2,3-dihydroxy-1-methylbenzene conflict with available information on the microbial metabolism of aromatic hydrocarbons. Marr and Stone (1961) postulated *trans*-1,2-dihydroxycyclohexa-3,5-diene as an intermediate in benzene degradation by *Pseudomonas aeruginosa*. Walker and Wiltshire (1953) isolated (+)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from culture filtrates of a *Bacillus* growing with naphthalene as the source of carbon. The same glycol was detected in the metabolism of naphthalene by five different microorganisms (Treccani *et al.*, 1954). Griffiths and Evans (1965) prepared a cell extract which, in the presence of a strong NADH-generating system, accumulated (+)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene. The same cell extract in the presence of NAD^+ oxidized this glycol to *cis-o*-hydroxybenzalpyruvate. The glycol formed from *p*-chlorotoluene is dehydrogenated in the presence of NAD^+ to 4-chloro-2,3-dihydroxy-1-methylbenzene which is not further metabolized. The enzyme catalyzing this reaction is presumably the same as the enzyme which is responsible for the dehydrogenation of *cis*-benzene glycol (Gibson *et al.*, 1968). It is proposed that the natural substrate, when *P. putida* is grown with toluene as sole carbon source, is *cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene.

When the organisms described by Walker and Wiltshire (1953) were grown on 1-chloronaphththalene, (+)-8-chloro-1,2 - dihydro - 1,2 - dihydroxynaphthalene was isolated from the culture filtrate (Walker and Wiltshire, 1955). Upon acidification, this glycol gave mainly 8-chloro-2-naphthol and a small amount of 8-chloro-1-naphthol. Analogous dehydration products were given by the glycol from *p*-chlorotoluene.

The metabolism of aromatic hydrocarbons and their halogenated derivatives by mammals has received considerable attention. Park and Williams (1953) showed that 20 times more catechol was produced from benzene than from phenol when rabbits were injected with these two compounds. These results suggested that perhaps there were two different pathways for the metabolism of benzene in rabbits. Subsequently Sato *et al.* (1963) showed that benzene was metabolized to *trans*-1,2-dihydroxycyclohexa-3,5-diene (*trans*-benzene glycol). Ayengar *et al.* (1959) purified an enzyme from rat liver which dehydrogenated this compound to catechol; $NADP^+$ was required as a cofactor in this reaction. The metabolism of naphthalene by rabbits proceeds through (+)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene, while the (−)-*trans* isomer is produced by rats (Booth and Boyland, 1949; Young, 1947).

Recent studies by Holtzman *et al.* (1967) have shown that mouse liver microsomes also oxidize naphthalene to *trans*-1,2-dihydro-1,2-dihydroxynaphthalene. When the experiment was performed in the presence of $^{18}O_2$ only one atom of isotopic oxygen was incorporated into the naphthalenediol. This observation provides evidence for the participation of 1,2-epoxynaphthalene as

an intermediate in naphthalene oxidation. Alternatively the incorporation of ^{18}O into 1,2-dihydro-1,2-dihydroxynaphthalene could be explained by the initial attack of a hydroxyl radical. Oxidation of the product formed followed by hydration of the resulting carbanion would give 1,2-dihydro-1,2-dihydroxynaphthalene. The diol thus formed would contain only one ^{18}O atom.

Chlorobenzene, when fed to rabbits, resulted in the isolation of (−)-*trans*-4-chloro-1,2-dihydroxycyclohexa-4,6-diene and 4-chlorocatechol (Smith *et al.*, 1950). The metabolism of (+)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene in rats resulted in the isolation of 9,10-dihydroxyphenanthrene which was isolated as its sulfate ester (Boyland and Sims, 1962a). In addition, phenanthrene is converted, in both rabbits and rats, into the (+) and (−) isomers of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene and *trans*-1,2-dihydro-1,2-dihydroxyphenanthrene (Boyland and Sims, 1962b).

It seems clear that, in the metabolism of aromatic hydrocarbons by mammals and the metabolism of polynuclear aromatic hydrocarbons by microorganisms, *trans*-glycols are the first detectable oxygenated intermediates. These observations can be explained by the prior formation of an epoxide; such a compound has been proposed in the metabolism of pyrene in rats (Boyland and Sims, 1964). Also, an epoxide was isolated when the insecticide heptachlor was fed to dogs (Davidow and Radomski, 1953). Although it has not been positively established, it seems likely that phenols arise in the animal body by the elimination of water from the dihydrodihydroxy compounds. The glycol isolated from *p*-chlorotoluene lost water very readily. After storing the crystals at 4° for 24 hr significant dehydration occurred.

Since the compound formed from *p*-chlorotoluene appears to have the *cis* configuration it is unlikely that it is formed from an epoxide precursor. However, at the present time the stereospecific enzymatic opening of an epoxide to give a *cis*-glycol cannot be discounted. The enzymatic formation of *cis*-glycols may be similar to the chemical hydroxylation of olefins by either dilute aqueous permanganate or hydrogen peroxide in the presence of osmium tetroxide. Another possiblility is that *cis* hydroxylation may be similar to photochemical oxidation; several transannular peroxides, both from biological and chemical sources, have been characterized (Vercauteren and Massart, 1962). A cyclic peroxide has been postulated in the enzymatic formation of catechol from anthranilic acid (Kobayashi *et al.*, 1964). Whether or not such a mechanism is present in the metabolism of mononuclear aromatic compounds awaits purification of enzyme systems and $^{18}O_2$ incorporation experiments. Such studies are in progress.

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