Formation of (+)-*cis*-2,3-Dihydroxy-1-methylcyclohexa-4,6-diene from Toluene by *Pseudomonas putida**

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ABSTRACT: Toluene is oxidized by a mutant strain of *Pseudomonas putida* (strain 39/D) to (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene. Acetylation of this product followed by condensation with maleic anhydride results in the formation of 1-methyl-2,3-diacetoxybicyclo(2,2,2)-7-hexene-5,6-dicarboxylic anhydride. The nuclear magnetic resonance spectrum of the condensation product establishes the *cis* configuration of the hydroxyl groups in the toluene oxidation product.

Washed cells of *P. putida* (parent strain) and cell extracts prepared from the same organism rapidly oxidize toluene, 3-methylcatechol, and (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene. The latter compound is enzymatically dehydrogenated by cell extracts, prepared from the parent organism, to form 3-methylcatechol. This reaction occurs under anaerobic conditions and oxidized nicotinamide–adenine dinucleotide is an essential cofactor for the reaction. The data presented provide evidence that (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene is an intermediate in the degradation of toluene by *P. putida*.

Toluene degradation by *Pseudomonas aeruginosa* has been studied by Kitagawa (1956). Cells of this organism were grown on nutrient broth and then exposed for several hours to small concentrations of toluene. The induced cells oxidized toluene, benzyl alcohol, benzaldehyde, benzoic acid, and catechol at approximately equal rates. These simultaneous adaptation experiments suggest that the initial attack on toluene by *P. aeruginosa* involves oxidation of the methyl group.

An alternative mode of attack was indicated by the sequential induction experiments of Claus and Walker (1964). A *Pseudomonas* sp. and an *Achromobacter* sp. were isolated by virtue of their ability to utilize toluene as sole source of carbon for growth. Both organisms, after growth on toluene, oxidized, without lag, toluene, benzene, catechol, 3-methylcatechol, and benzyl alcohol. Benzaldehyde and benzoic acid were metabolized after a lag period. 3-Methylcatechol was detected chromatographically in the culture medium and it was suggested that this compound is an intermediate in toluene degradation.

More recently Gibson *et al.* (1968a) obtained similar results with toluene-grown cells of *P. putida*. 3-Methylcatechol was isolated from culture filtrates of growing cells and shown to be identical with a synthetic sample of the same compound. Cultures of *P. putida*, after grown with toluene, oxidized *p*-chlorotoluene to (+)-*cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene and 4-chloro-2,3-dihydroxy-1-methylbenzene (Gibson *et al.*, 1968b). It was suggested that the true intermediate in the degradation of toluene by *P. putida* is *cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (*cis*-2,3-dihydro-2,3-dihydroxytoluene).

We now wish to report the accumulation of *cis*-2,3-dihydro-2,3-dihydroxytoluene by a mutant strain of *P. putida*. The identification of this compound as the *cis* isomer distinguishes its enzymatic formation from all previous reports on the oxidation of aromatic hydrocarbons.

Materials and Methods

Analytical Methods. Ultraviolet spectra were determined on a Cary Model 14 recording spectrophotometer. Infrared absorption spectra were recorded on a Beckman IR-5A double-beam recording spectrophotometer. Mass spectra were determined on a Consolidated Engineering Co. 21102, modified, mass spectrometer. The 60- and 100-Mc nuclear magnetic resonance spectra were recorded on Varian A-60 and HA-100 spectrometers, respectively. Tetramethylsilane was used as an internal reference for all nuclear magnetic resonance spectra. Melting points were obtained by use of a Fisher-Johns melting point apparatus and are uncorrected. Thin-layer chromatography was performed using Eastman Chromatogram sheets, type K130R (silica gel with fluorescent indicator). Solvents used for chromatography were (a) benzene–methanol (95:5, v/v) and (b) chloroform–acetone (80:20, v/v). Compounds were located on chromatograms by the use of ultraviolet light and also by spraying with a 2% (w/v) solution of 2,6-dichloroquinone-4-chloroimide. Oxygen consumption was measured by conventional manometric techniques.

Organism and Growth Conditions. P. putida was grown at 30° in the mineral salts medium of Stanier *et al.* (1966). Toluene was supplied to 500-ml cultures, in 2-l. erlenmeyer flasks, by placing 2 ml in a glass tube which was suspended above the medium by a neoprene stopper. The open end of the tube above the stopper was plugged with cotton. A hole in the glass tube, below the stopper, allowed toluene to diffuse into the culture flask. Cultures were aerated on a reciprocal shaker. Yeast extract medium was prepared by the addition of Difco yeast extract (5 g/l.) to the mineral salts

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medium. Unless stated otherwise all other substrates were added to mineral salts medium at a concentration of 2 g/l.

Isolation of Mutant Strains of P. putida. The procedure used was that of Ornston (1966). Colonies of the parent strain which grew on 10 mM succinate but not on toluene were marked at mutants. Each strain was grown on yeast extract medium in the presence of toluene vapor. Samples of each culture filtrate were extracted with ethyl acetate and chromatographed in solvent b. Out of 113 strains examined four were found to accumulate (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene in the culture medium. One of these was labeled *P. putida* 39/D and used in the present work.

Isolation of (+)-cis-2,3-Dihydroxy-1-methylcyclohexa-4,6-diene (Compound I). P. putida 39/D was grown in 2 l. of glucose medium (four 2-l. erlenmeyer flasks). Toluene was supplied in the vapor phase as described above. The flasks were shaken on a reciprocal shaker for 30 hr. The cells were removed by centrifuging at 15,000*g* for 30 min. The clear supernatant solution was evaporated to dryness under vacuum at 40°. Methanol extraction of the residue, followed by removal of the solvent, gave 2.42 g of a yellow oil. The oil was dissolved in chloroform and applied to the top of a silica gel column (3 × 50 cm). After washing the column with chloroform compound I was eluted with 800 ml of chloroform–methanol (99.5:0.5). Two crystallizations from petroleum ether (30–60°) gave 1.94 g of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (compound I): mp 59°; $[\alpha]_D$ +25° (*c* 0.4, MeOH); $\lambda_{max}^{H_2O}$ 265 mμ (ε 5220); λ_{max}^{Nujol} 3.12, 6.06, and 6.25 μ. *Anal.* Calcd for $C_7H_{10}O_2$ (126): C, 66.66; H, 7.93. Found: C, 66.53: H, 7.80.

Trimethylsilyation of Compound I. Compound I (40 mg) was dissolved in 3 ml of dry pyridine and treated with 0.5 ml each of hexamethyldisilazane and trimethylchlorosilane. The resulting mixture was evaporated to dryness at room temperature. The residue was dissolved in 20 ml of carbon tetrachloride and the solution was filtered. After removing the solvent under vacuum, the residue was dissolved in carbon tetrachloride and used for nuclear magnetic resonance studies.

1-Methyl-2,3-diacetoxybicyclo(2,2,2)-7-hexene-5,6-dicarboxylic Anhydride (Compound IA). Compound I (126 mg) was dissolved in 1.5 ml of pyridine. To this mixture 1.0 ml of acetic anhydride was added and the resulting solution was allowed to stand at room temperature for 15 hr. Removal of the solvent gave 205 mg of a colorless oil. The oil was dissolved in 5 ml of dry benzene which contained 100 mg of maleic anhydride. After 6 days at room temperature the solvent was removed and the residue was crystallized from acetone–petroleum ether (30–60°). Two crystallizations gave 236 mg of IA, mp 196–197°. *Anal.* Calcd for $C_{15}H_{16}O_7$ (308): C, 58.44; H. 5.19. Found: C, 58.72; H, 5.34.

1-Methyl-2,3-diacetoxybicyclo(2,2,2)-hexane-5,6-dicarboxylic Anhydride (Compound IB). Compound IA (103 mg) was hydrogenated at atmospheric pressure in ethyl acetate (25 ml) containing 0.5 ml of acetic anhydride. Prehydrogenated palladium–charcoal (5%, 55 mg) was used as a catalyst. The hydrogenation was complete in 5 min with the uptake of 1 mole of hydrogen/mole of IA. The mixture was filtered and the solvent was removed under vacuum. Two crystallizations from acetone–petroleum ether (30–60°) gave 78 mg of IB, mp 190–191°. *Anal.* Calcd for $C_{15}H_{18}O_7$ (310): C, 58.06; H, 5.81; Found: C, 58.19; H, 5.94.

Acid-Catalyzed Dehydration of Compound I. Compound I was dissolved in 10 ml of 1 N HCl and heated on a steam bath for 5 min. The solution was cooled and extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. The solvent was removed to leave a yellow oil, which was dissolved in benzene and passed through a silica gel column (1 × 30 cm). Removal of the solvent after column chromatography gave a pale yellow oil. The infrared spectrum of the isolated product was identical with the spectrum given by synthetic *o*-cresol.

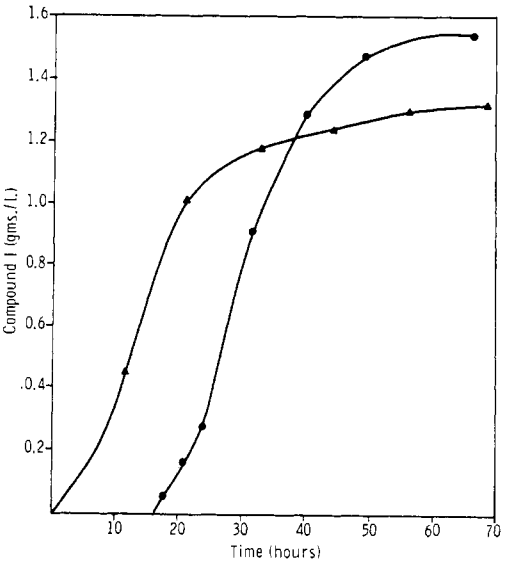


FIGURE 1: Production of compound I by *P. putida* 39/D. Culture flasks (500 ml) contained 100 ml of mineral salts medium and glucose 2 g/l., or yeast extract, 5 g/l. Toluene was supplied in the vapor phase. Samples were taken at time intervals and centrifuged to remove the cells, 0.05 ml of the clear supernatant solution was diluted to 3.0 ml with distilled water and the absorbance was measured at 265 mμ. The amount of compound I present was calculated using $\epsilon_{265\ m\mu}$ 5220; (▲—▲—▲) glucose; (●—●—●) yeast extract.

All other materials and methods are as previously described (Gibson *et al.*, 1968b).

Results

When *P. putida* 39/D was grown on yeast extract medium, in the presence of toluene, a neutral ultraviolet-absorbing compound was excreted into the culture medium. Ethyl acetate extracts of the culture filtrate were chromatographed in solvent systems a and b. A single compound was detected; R_F 0.25 and 0.43 in solvents a and b, respectively. Culture filtrates were also acidified with 5 N HCl and subjected to the same chromatographic treatment. This procedure caused the disappearance of compound I with the resultant formation of a product which was chromatographically identical with *o*-cresol. When the organism was grown in the absence of toluene no products were detected in the culture medium.

Glucose was used as a carbon source for the conversion of toluene into I. When yeast extract was used as the growth substrate an 18-hr lag period was observed before I appeared in the culture filtrate (Figure 1).

Maximum yields of I were obtained at pH 7.5. Below pH 7.0 I dehydrated to give *o*-cresol.

Identification of I. From ultraviolet and infrared spectra

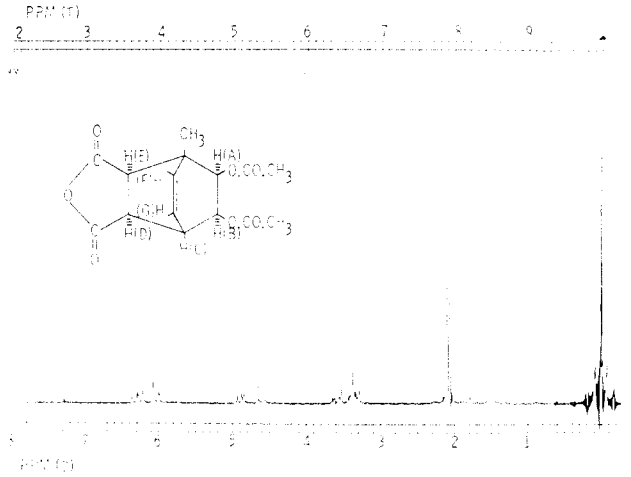


FIGURE 2: Nuclear magnetic resonance spectrum of 1-methyl-2,3-diacetoxybicyclo(2,2,2)-7-hexene-5,6-dicarboxylic anhydride (IA). The sample was dissolved in deuterated chloroform and the spectrum recorded at 100 Mc. Tetramethylsilane was used as an internal standard.

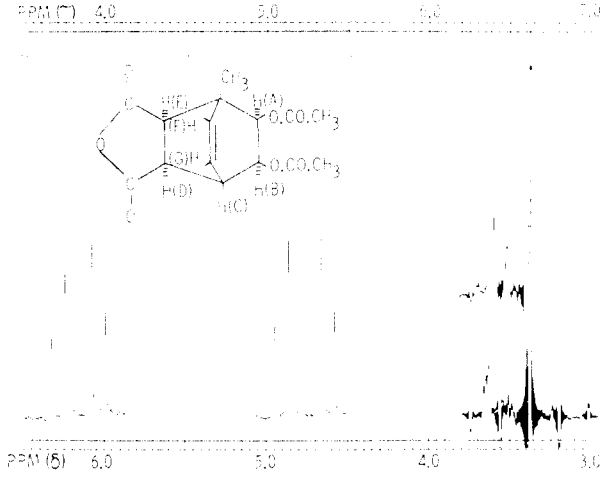
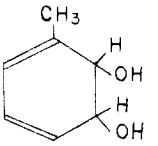


FIGURE 3: Nuclear magnetic resonance spectrum of 1-methyl-2,3-diacetoxybicyclo(2,2,2)-7-hexene-5,6-dicarboxylic anhydride (IA) between δ 4.5 and 6.5. Coupling of H(C) destroyed by irradiation at δ 3.38.

I appeared to be a hydroxylated cyclohexadiene derivative. The ready dehydration to give *o*-cresol suggested that I was a dihydrodihydroxy derivative of toluene. Further evidence was provided by the mass spectrum (Table I) which showed a small parent ion at *m/e* 126. The base peak at *m/e* 108 and all other fragments are identical with those given by a synthetic sample of *o*-cresol.

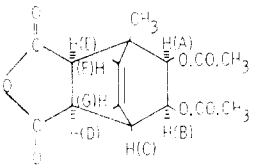
The nuclear magnetic resonance spectrum (in deuterated acetone) showed bands at δ 1.87, 3 H (methyl group); 3.6, 2 H (adjacent OH groups, disappeared on shaking with D_2O); 3.91, 1 H (unsymmetrical broad doublet, J = 6.5 cps); 4.18, 1 H (unsymmetrical broad doublet, J = 7.0 cps); 5.71, 3 H (multiplet, olefinic protons). In order to resolve the signals at δ 3.91 and 4.18 compound I was treated with trimethylchlorosilane and hexamethyldisilazane. The nuclear magnetic resonance spectrum of the silyated product in carbon tetrachloride showed bands at δ 1.82, 3 H (methyl group); 3.81, 1 H (doublet, J = 5.5 cps); 4.12, 1 H (broad doublet, J = 5.5 cps); 5.64, 3 H (multiplet, olefinic protons). Spin decoupling by irradiation at the center of the signals due to the olefinic protons sharpened the bands at δ 3.81 and 4.12 into a typical AB system, J for each doublet was 5.5 cps.

These results establish the following structure for I.



I, 2,3-dihydroxy-1-methylcyclohexa-4,6-diene

From the above data it was not possible to assign a *cis* or *trans* configuration to the hydroxyl groups. This is because the observed coupling constants between the protons attached to the carbon atoms which also carry the oxygen atoms are equivocal to the bond angle for either a *cis* or *trans* orientation. In addition conformational rotation affecting bond angles has to be taken into consideration. In order to clarify this point I was acetylated and then condensed with maleic anhydride to form 1-methyl-2,3-diacetoxybicyclo(2,2,2)-7-hexene-5,6-dicarboxylic anhydride (IA). The nuclear magnetic resonance



Compound IA

spectrum of this compound in deuterated chloroform is shown in Figure 2. Signals were detected at δ 1.52, 3 H (methyl group); 2.07 and 2.10, 6 H (two acetate methyl groups); 3.33, H(E), broad doublet, J_{ED} = 9.0 cps; 3.38, H(C), multiplet; 3.58, H(D), double doublet, J_{DE} = 9.0 cps, J_{DC} = 3.0

TABLE I: Mass Spectrum of Compound I.[a]

m/e	% Base Peak	*m/e*	% Base Peak
50	5	78	8.3
51	7.5	79	28.4
52	3.3	80	10.0
53	6.7	89	8.3
54	3.3	90	20.0
63	5.0	91	6.7
65	1.7	108	100.0
77	23.4	109	5.0
		126	8.1

[a] The spectrum was run on a Consolidated Engineering Company modified mass spectrometer; inlet reservoir temperature 150°, ion source 250°, ionizing voltage 70 eV, and ionizing current 50 μA.

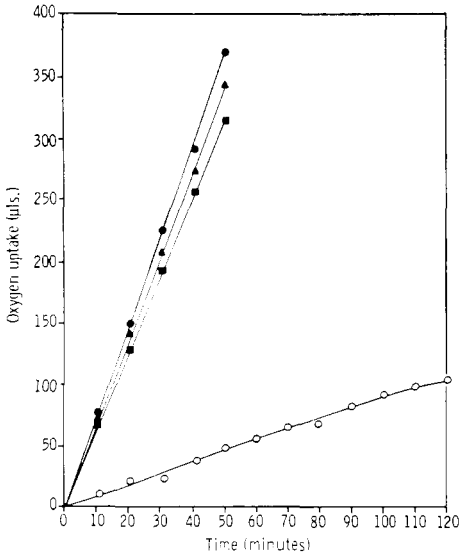


FIGURE 4: Oxidation of toluene and related compounds by *P. putida* (wild type) and *P. putida* (strain 39/D). Warburg flasks contained in a final volume of 3.0 ml; KH_2PO_4 buffer, 160 µmoles; cell suspension, 1.0 ml; and substrate, 5 µmoles in 0.2 ml of *N,N*-dimethylformamide. Results are corrected for endogenous respiration in the absence of substrate: *P. putida* (wild type), (●—●—●) 3-methylcatechol, (▲—▲—▲) compound I, (■—■—■) toluene; *P. putida* (strain 39/D), (○—○—○) toluene.

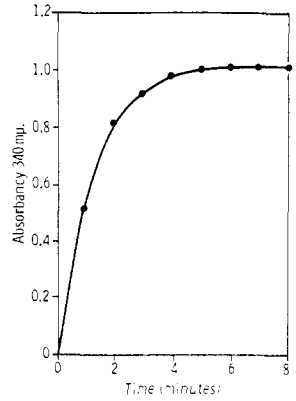
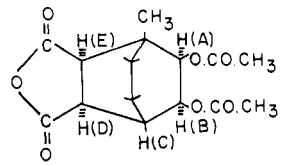


FIGURE 5: Dehydrogenation of compound I by cell extract. Anaerobic cuvets contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 250 µmoles; NAD^+, 1.0 µmole; compound I, 0.5 µmole: and cell extract, 4.7 mg of protein. A reference cuvet contained all components except compound I.

cps; 4.62, H(A), doublet, J_{AB} = 8.5 cps; 4.92, H(B), double doublet, J_{BA} = 8.5 cps, J_{BC} = 3.0 cps; 6.05, H(F), double doublet J_{FG} = 8.0 cps, J_{FC} = 1.0 cps; 6.28, H(G) double doublet, J_{GF} = 8.0 cps. J_{GC} = 7.0 cps. The coupling of H(C) with H(B) and H(G) was destroyed by irradiation at δ 3.38 (Figure 3). This treatment resolved the coupling between H(A) and H(B) into a typical AB spectrum, J_{AB} = J_{BA} = 8.5 cps. Similarly the coupling between the olefinic protons was also converted into an AB system, J_{FG} = J_{GF} = 8.5 cps. The identical coupling constants between H(A), H(B), and H(F), H(G) do not permit absolute assignment of the chemical shifts of these protons. In order to unequivocally establish the position of H(A) and H(B) in the nuclear magnetic resonance spectrum, compound IA was hydrogenated. The nuclear magnetic resonance spectrum of the hydrogenated product (IB) showed signals at δ 1.18, 3 H (methyl group); 1.58, 4 H (methylene protons on cyclohexane ring); 2.05 and 2.08, 6 H (two acetate methyl groups); 2.40, H(C),



Compound IB

multiplet; 3.20, H(E), J_{ED} = 10.0 cps; 3.52, H(D), double doublet, J_{DE} = 10.0 cps; J_{DC} = 3.0 cps; 4.86, H(A), doublet, J_{AB} = 8.5 cps; 5.17, H(B) double doublet, J_{BA} = 8.5 cps, J_{BC} = 3.0 cps. This nuclear magnetic resonance data establishes that the signals observed at δ 6.05 and 6.28 (Figure 3) are due to the olefinic protons in compound IA. In addition the observed coupling constants (8.5 cps) between H(A) and H(B) in compounds IA and IB show that these vicinal protons are *cis* to each other and confirm that the structure of the diol produced from toluene by *P. putida* is (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (see Discussion).

Experiments with Washed Cell Suspensions. P. putida (wild type) was grown with glucose as carbon source. Toluene was supplied in the vapor phase. Cells were harvested in the logarithmic phase of growth (12 hr) and washed three times with 0.5 M KH_2PO_4 buffer, pH 7.2. The washed cells oxidized toluene, compound I, and 3-methylcatechol at approximately the same rate (Figure 4). Washed cell suspensions of *P. putida* 39/D, grown under the same conditions, oxidized toluene at 15% of the rate observed with the wild-type organism. One mole of oxygen per mole of toluene was utilized. Chromatographic examination of the reaction mixture revealed the presence of I. The same cell suspension did not oxidize I; however 3-methylcatechol was oxidized at approximately the same rate which was observed with *P. putida* (wild type).

Experiments with Cell Extract. Cell extracts were prepared from *P. putida* (wild type) as described previously (Gibson *et al.*, 1968a). Toluene, compound I, and 3-methylcatechol were oxidized with the consumption of 2.0, 1.5, and 1.0 moles of oxygen per mole of substrate, respectively. Extracts prepared from *P. putida* 39/D oxidized toluene extremely slowly and did not oxidize I. The same cell extract oxidized 3-methylcatechol with the consumption of 1 mole of oxygen/mole of substrate.

Cell extracts were prepared from *P. putida* (wild type) grown with toluene as carbon source and incubated anaerobically with compound I and NAD^+.[1] A stoichiometric reduction of NAD^+ was observed (Figure 5). At the end of the reaction the mixture was acidified with 5 N H_2SO_4 and the precipitated protein was removed by centrifugation. The clear supernatant solution was extracted with ethyl acetate. Chromatography of the organic extract in solvents a and b revealed that I was dehydrogenated to form 3-methylcatechol.

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

When $NADP^+$ was used as electron acceptor no activity was observed.

Discussion

Although the microbial degradation of oxygenated aromatic compounds is well documented (Gibson, 1968), relatively little is known about the metabolism of mononuclear aromatic hydrocarbons. Toluene degradation by microorganisms may proceed by oxidation of the methyl group (Kitagawa, 1956; Nozaka and Kusunose, 1968) or by hydroxylation of the aromatic nucleus to form 3-methylcatechol (Claus and Walker, 1964; Gibson *et al.*, 1968a; Nozaka and Kusunose, 1969). In order to elucidate the mechanism of oxygen fixation into the aromatic nucleus it is important that the identities of the initial oxidation products be clearly established.

The compound produced from toluene by *P. putida* 39/D was initially recognized as a dihydrodiol derivative from spectroscopic data and the observation that acid-catalyzed dehydration produced *o*-cresol. The nuclear magnetic resonance spectrum of the isolated diol does not allow an absolute determination of the position of the hydroxyl groups. This is because the dihedral angle between the vicinal protons on the carbon atoms carrying the hydroxyl groups give rise to coupling constants which are indicative of either a *cis* or a *trans* configuration. In addition interpretation is complicated by the possible conformational changes in the molecule. On the other hand, the Diels–Alder adduct (IA) is a rigid system which permits assignment of the relative orientation of the hydroxyl groups. If the hydroxyl groups have the *trans* configuration the dihedral angle between the vicinal protons will be $\sim 120°$ and the coupling constants will be ~ 2 cps. A *cis* configuration gives a dihedral angle $\sim 0°$ for the vicinal protons on the carbon atoms carrying the hydroxyl groups and coupling constants ~ 8 cps. (Karplus, 1963). The observed coupling constants for H(A) and H(B) (Figure 3) are 8.5 cps and establish the structure of the diol formed from toluene as (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene.

Arene oxides have been proposed as intermediate compounds in the formation of phenols, dihydrodiols, and premercapturic acids (Jerina *et al.*, 1968). The formation of *cis*-2,3-dihydro-2,3-dihydroxytoluene suggests that 2,3-toluene oxide is unlikely to be an intermediate in the enzymatic degradation of toluene by *P. putida*. However at this time the enzymatic sterospecific opening of an epoxide to give a *cis*-dihydrodiol cannot be discounted. We are at present investigating the possibility that *cis* hydroxylation may be similar to photochemical oxidation and oxidations by singlet oxygen.

The observations that *cis*-2,3-dihydro-2,3-dihydroxytoluene is oxidized by *P. putida* at the same rate as toluene and 3-methylcatechol supports the contention that this compound is an intermediate in the degradation of toluene. Further support is provided by the ready stoichiometric dehydrogenation of *cis*-2,3-dihydro-2,3-dihydroxytoluene to 3-methylcatechol by cell extracts. It is of interest to note that NAD^+ is a specific electron acceptor for this reaction, $NADP^+$ being inactive. An $NADP^+$-dependent enzyme which catalyzes the dehydrogenation of *trans*-1,2-dihydroxy-1,2-dihydroxybenzene to catechol, has been purified from rabbit liver (Ayengar *et al.*, 1959).

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