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Bacterial Metabolism of *para*- and *meta*-Xylene: Oxidation of the Aromatic Ring

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Pseudomonas putida 39/D oxidized *p*-xylene to *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (*cis*-*p*-xylene dihydrodiol). The latter compound was isolated in crystalline form and its physical properties were determined. The *cis* configuration of the hydroxyl groups in the oxidation product was inferred from its ability to form an isopropylidene derivative with 2,2-dimethoxypropane. Acid treatment of *cis*-*p*-xylene dihydrodiol resulted in the formation of 2,5-dimethylphenol. A partially purified preparation of *cis*-toluene dihydrodiol dehydrogenase oxidized *cis*-*p*-xylene dihydrodiol to 1,2-dihydroxy-3,6-dimethylbenzene (3,6-dimethylpyrocatechol). *P. putida* 39/D oxidized *m*-xylene to a compound whose spectral and chromatographic characteristics were consistent with the structure 3,5-dimethyl-3,5-cyclohexadiene-1,2-diol. This product was very unstable, and all attempts to isolate it led to the formation of 2,4-dimethylphenol.

The bacterial oxidation of *p*- and *m*-xylene may be initiated by oxidation of one of the two methyl groups or by direct oxidation of the aromatic nucleus (1). Bacteria that can utilize either of the two xylene isomers as carbon and energy sources for growth appear to oxidize these substrates through the corresponding toluic acids to methyl-substituted catechols. The latter compounds are the substrates for enzymatic fission of the aromatic nucleus (2, 5, 6). However, there are certain microorganisms that, even though they cannot utilize *p*- and *m*-xylene as growth substrates, are able to oxidize these compounds to an appreciable extent. These observations are the result of Raymond and Jamison's elegant investigations into the co-oxidation potential of different *Nocardia* species (17). Thus *Nocardia salmonicolor* A-100, *N. corallina* A-6, *N. corallina* A-11, and *N. minima* A-138, when grown on hexadecane in the presence of *p*-xylene, accumulated *p*-toluic acid and 2,3-dihydroxy-*p*-toluic acid in the culture medium (18). Under the same cultural conditions, the above *Nocardia* strains did not oxidize *m*-xylene. A different strain (V-49) of *N. corallina*, in addition to oxidizing *p*-xylene to *p*-toluic acid and 2,3-dihydroxy-*p*-toluic acid, also produced 3,6-dimethylpyrocatechol and α,α'-dimethyl-*cis*, *cis*-muconic acid (13). The pathway proposed for the formation of the latter two compounds is shown in Fig. 1.

We now wish to report the isolation and identification of the initial oxidation products formed from *p*- and *m*-xylene by *Pseudomonas putida* 39/D. This organism is a mutant strain that is known to oxidize several different aromatic hydrocarbons to *cis*-dihydrodiols (8).

MATERIALS AND METHODS

Materials. All chemicals were of the highest purity commercially available. *p*-Xylene (99.95%) and *m*-xylene (99.92%) were from Phillips Petroleum Co. Beef heart lactic acid dehydrogenase, (L-lactate: nicotinamide adenine dinucleotide (NAD^+) oxidoreductase; EC 1.1.1.27), sodium pyruvate, and NAD^+ were from Sigma Chemical Co. 2,5-Dimethylphenol was a gift from P. J. Chapman, Department of Biochemistry, University of Minnesota. Silica Gel 60 (Brinkmann Instruments Inc.) was used for column chromatography.

Analytical methods. Ultraviolet and visible spectra were determined on Cary model 14 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer. Crystalline samples were mulled in Nujol and placed between NaCl plates. Noncrystalline samples were run on neat liquid films between NaCl plates. All absorptions were referenced to the absorptions of polystyrene. Low-resolution mass spectra were determined on a DuPont-Consolidated Electrodynamics Corp. model 21-491 mass spectrometer. Parent ion molecular weights were determined by peak matching with assigned perfluoroalkane peak fragments. The determinations were made on a Dupont-Consolidated Electrodynamics Corp. model 21-110 high-resolution mass spectrometer. Proton magnetic resonance (PMR) spectra were recorded on a Perkin-Elmer R-12 spectrometer. Absorptions were assigned δ values at the midpoint of half-height and are referenced to Me_4Si. Melting points were obtained by use of a

FIG. 1. *Proposed pathway for the oxidation of p-xylene to α,α'-dimethylmuconic acid by N. corallina V-49* (*13*).

Büchi melting-point apparatus and are uncorrected. Thin-layer chromatography was performed by using Eastman Chromatogram Sheets, type K130R (silica gel with fluorescent indicator). The solvent used for chromatography was chloroform-acetone (80:20, vol/vol; solvent A). Compounds were located on chromatograms by spraying with a 2% (wt/vol) solution of 2,6-dichloroquinone-4-chloroimide (Gibb's reagent) and also by the use of ultraviolet light.

Microorganisms. *P. putida* (wild type) was orginally isolated by virtue of its ability to utilize ethylbenzene as sole source of carbon and energy for growth (12). This organism will also utilize benzene and toluene as growth substrates. However, none of the three xylene isomers would support growth. *P. putida* 39/D was isolated after treatment of the wild-type strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10). This organism oxidizes benzene (9), toluene (11, 15, 21), ethylbenzene (10), 1-phenylethanol (12), acetophenone (10), *p*-chloro-, *p*-fluoro-, and *p*-bromotoluene, chlorobenzene, and cyanobenzene (21) to *cis*-dihydrodiol derivatives.

Growth conditions. *P. putida* (wild type) was grown on toluene as described previously (11). *P. putida* 39/D and *P. putida* (wild type) were grown in mineral salts medium (20) containing 0.2% succinate and either *p*-xylene or *m*-xylene.

Isolation of cis-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (compound I). *P. putida* 39/D was grown in 10 liters of succinate-mineral salts medium in a New Brunswick M14 fermentor. *p*-Xylene was supplied to the medium as described previously for ethylbenzene (10). The accumulation of compound I in the culture medium was monitored by measuring the increase in absorbance of the culture filtrate at 270 nm. After 8 h, the amount of compound I present in the culture medium, calculated by using $\epsilon = 6{,}500$, was 1.89 g. Incubation overnight for a further 12 h did not lead to an increase in the production of compound I. The cells in the culture were removed by centrifugation, and the clear supernatant solution was extracted with 4 liters of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to leave 1.4 g of a brown solid. This residue was dissolved in hot benzene and decolorized with active charcoal. The clear benzene solution was concentrated to a small volume, and petroleum ether (40 to 60 C) was added until the solution began to crystallize. Three recrystallizations from benzene-petroleum ether gave 653 mg of white needles, mp 77 to 78 C.

Acid-catalyzed dehydration of compound I. Compound I (35 mg) was dissolved in 15 ml of anhydrous ether. Concentrated HCl (10 μliters) was added to the solution. After 15 min the ether solution was washed successively with 15 ml of water, 10% sodium bicarbonate, and water. After drying over anhydrous sodium sulfate, the ether was removed to give 28 mg of compound IA as a white solid, mp 75 C.

Preparation of 1,3-dioxolo-2,2-dimethyl-8H, 9H,4,7,-dimethyl-cyclohexa-4,6-diene (compound IB). Compound I (80 mg) was dissolved in 10 ml of 2,2-dimethoxypropane. The solution was cooled in an ice bath before the addition of 1.0 mg of *p*-toluene sulfonic acid. After 20 min, 2.0 g of Na_2CO_3 and 10.0 ml of benzene were added to the reaction mixture. The Na_2CO_3 was removed by filtration, and the solvent was removed to leave 71 mg of a brown oil. Thin-layer chromatography in solvent A revealed the presence of 2,5-dimethylphenol (R_f, 0.54) and a new product (R_f, 0.58). The xylenol was removed by dissolving the oil in $CHCl_3$ and passing it through a column (10 by 1.0 cm) of basic alumina. Removal of the $CHCl_3$ gave 33 mg of a pure sample of compound IB.

Isolation of 1,2-dihydroxy-3,6-dimethylbenzene (compound II). Cells of *P. putida* (wild type), after growth on toluene, were suspended in 0.05 M KH_2PO_4 buffer, pH 7.2 (1.0 g [wet weight] of cells per 3.0 ml of buffer), and disrupted with a model LS-75 sonifier (Branson Ultrasonics Corp., Stanford, Conn.). The suspension was centrifuged at 38,000 × *g* for 1 h, and the clear supernatant liquid was taken as a source of cell extract. Extracts prepared in this manner have previously been demonstrated to contain an enzyme that catalyzes the NAD^+-dependent oxidation of *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (*cis*-toluene dihydrodiol) to form 3-methylcatechol (11). The cell extract (11,220 U; specific activity, 1.5) was applied to the top of a diethylaminoethylcellulose-Sephadex A50 column (56 by 4 cm), which was then washed with 0.05 M KH_2PO_4 buffer (pH 7.2) until protein could no longer be detected in the eluate. At this point, a linear gradient of 0.0 to 0.5 M KCl in the above buffer was applied to the column and 18-ml fractions were collected. *cis*-Toluene dihydrodiol dehydrogenase (3,623 U; specific activity, 11.2) was eluted between 0.25 and 0.30 M KCl. This preparation, which was almost completely free from catechol-2,3-oxygenase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.1.2), was used in the following experiment. The reaction was performed in a 250-ml Buchner flask containing

50 ml of 0.05 M KH_2PO_4, pH 7.2, to which the following reactants were added (in millimoles); *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (compound I), 0.6; sodium pyruvate, 1.0; NAD^+, 0.03; lactate dehydrogenase, 5.0 ml of protein, 2,800 U; and partially purified *cis*-toluene dihydrodiol dehydrogenase, 8.0 mg of protein, 89.6 U. The flask was alternately evacuated and flushed with nitrogen several times. The reaction was allowed to proceed under nitrogen, with stirring, for 6 h. At this time 5.0 ml of 5 N H_2SO_4 was added to the reaction mixture. The flask contents were extracted twice with two 50-ml volumes of ethyl acetate. After drying over anhydrous sodium sulfate, the solvent was removed to leave 61 mg of a white solid. This residue was dissolved in chloroform and applied to the top of a silica gel column (5.0 by 0.5 cm). The column was eluted with chloroform, and 3.0-ml fractions were collected. Fractions 5 through 11 were pooled and the solvent was removed in vacuo. The residue was crystallized from hot chloroform to give 42 mg of white needles (compound II) that melted at 102 to 103 C.

RESULTS

p-Xylene metabolism: detection and isolation of compound I. *P. putida* 39/D was grown on succinate mineral salts medium in the presence of *p*-xylene. Samples (1.0 ml) were taken throughout the growth period and analyzed by ultraviolet spectrophotometry and thin-layer chromatography. After 3 h a product was detected in the culture filtrate that gave the absorption spectrum shown in Fig. 2. Thin-layer chromatography in solvent A revealed the presence of a single ultraviolet-absorbing compound (R_f, 0.23) that gave a brown color on heating with Gibb's reagent. When the culture filtrate was acidified before analysis by thin-layer chromatography, the compound with R_f 0.23 (compound I) disappeared and was replaced by a less polar product (compound IA), which gave an R_f of 0.53 in solvent A. Compound IA absorbed ultraviolet light and gave an immediate pink color with Gibb's reagent. Compound I was isolated as white crystalline needles from 10 liters of culture filtrate (see Materials and Methods). In a control experiment, *P. putida* 39/D was grown on glucose mineral salts medium in the absence of *p*-xylene. No products were detected in the culture filtrate.

Identification of compound I. The white needles obtained from the oxidation of *p*-xylene by *P. putida* 39/D had the following physical properties: mp 77 to 78 C; λ_{max}^{MeOH}, 270 nm (ϵ = 6,500); λ_{max}^{Nujol}, 3.01, 6.01, 6.17, 6.30, and 11.95 μ. The 60-MHz PMR spectrum of compound I in deuterated acetone (Fig. 3) showed single bands at δ, 1.87, 6H (two methyl-group protons); 3.55, 2H (adjacent OH protons, disappears on shaking with D_2O); 4.03, 2H (hydroxymethine protons); and 5.58, 2H (olefinic protons). Compound I proved to be very unstable, and after standing at room temperature for 6 h was converted almost completely to compound IA. This observation was confirmed by mass spectral analysis where the parent ion was observed at *m/e* 122 (P-18). The spectrum was identical to that given by a synthetic sample of 2,5-dimethylphenol. The facile dehydration of compound I to give 2,5-dimethylphenol was confirmed by acid-catalyzed dehydration (see Materials and Methods). The product obtained showed the same chromatographic properties as compound IA and gave an infrared spectrum identical to that given by an authentic sample of 2,5-dimethylphenol. In addition, the dehydration product and 2,5-dimethylphenol both melted at 75 C. A mixed melting point showed no depression. The above results establish the structure of compound I as 3,6-dimethyl-3,5-cyclohexadiene-1,2-diol. Evidence for the *cis*

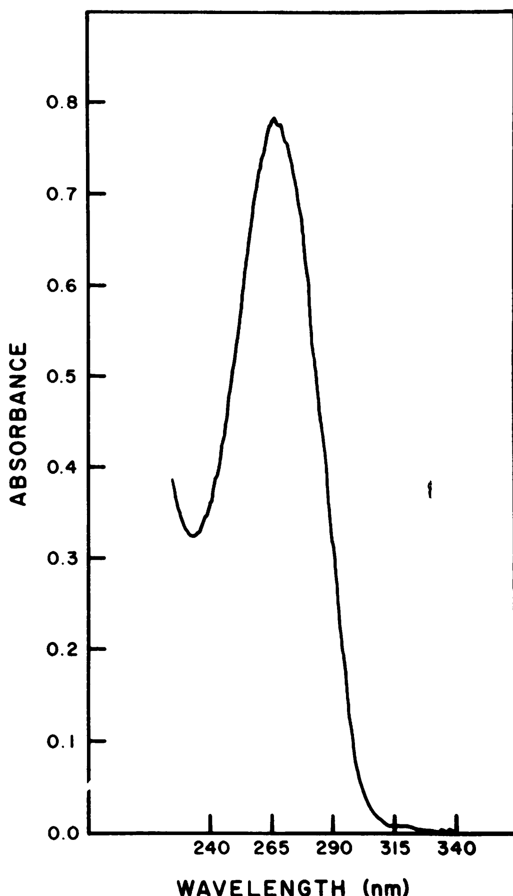

FIG. 2. *Absorption spectrum of cis-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (compound I) formed from p-xylene by P. putida 39/D.*

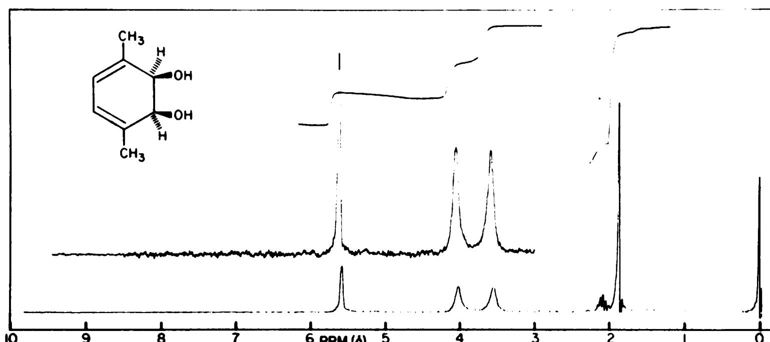

FIG. 3. *Proton magnetic resonance spectrum of cis-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol. The sample was dissolved in deuterated acetone, and the spectrum was recorded at 60 MHz. Tetramethylsilane was used as the internal standard.*

configuration of the hydroxyl groups was obtained by the reaction of compound I with 2,2-dimethoxypropane to give an isopropylidene derivative (compound IB). This product, which was obtained as a colorless oil, had the following properties: analysis, calculated mass for $^{12}C_{11}{}^{1}H_{16}{}^{16}O_2$, 180.1150, found mass 180.1149; λ_{max}^{oil}, 6.05, 6.25, and 7.35 μ; λ_{max}^{MeOH}, 254 nm (ϵ = 2,900). The 60-MHz PMR spectrum of compound IB in CCl_4 (Fig. 4) gave single bands at δ, 1.31, 3H (methyl); 1.38, 3H (methyl): 1.89, 6H (two methyl); 4.40, 2H (alkoxymethine); and 5.58, 2H (olefinic). These results identify compound IB as 1,3-dioxolo-2,2-dimethyl-8H,9H,4,7-dimethylcyclohexa-4,6-diene and supports the assignment of a *cis* configuration of the hydroxyl groups in the *p*-xylene oxidation product, 3,6-dimethyl-3,5-cyclohexadiene-1,2-diol.

Enzymatic oxidation of cis-3,6-dimethyl-3,5-cyclohexadiene-1,2. *P. putida* 39/D would not oxidize compound I. However, a partially purified preparation of diol dehydrogenase obtained from the wild-type strain, when incubated with *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol under anaerobic conditions led to the formation of 1,2-dihydroxy-3,6-dimethylbenzene (compound II). This compound was isolated in crystalline form. Its properties (mp 102 to 103 C; λ_{max}^{MeOH}, 275 nm (ϵ = 1,450); λ_{max}^{Nujol}, 2.95, 6.66, 7.04, and 12.46 μ) were consistent with the proposed structure. Further support was provided by the 60-MHz PMR spectrum in deuterated chloroform. Single peaks were observed at δ, 2.18, 6H (two methyl-group protons); 5.05, 2H (hydroxyl protons); and 6.57 2H (aromatic protons).

Oxidation of m-xylene by P. putida 39/D. When *P. putida* 39/D was grown on succinate-mineral salts medium in the presence of *m*-xylene, a neutral compound that showed maximal absorption at 270 nm was excreted into the culture medium. Thin-layer chromatography of an ethyl acetate extract of the culture filtrate, in solvent A, revealed the presence of a compound (R_f, 0.25) that gave a brown color on heating with Gibb's reagent (compound III). All attempts to isolate and identify this product

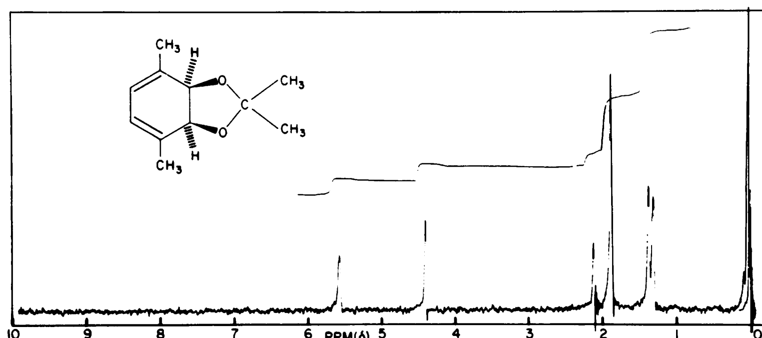

FIG. 4. *Proton magnetic resonance spectrum of 1,3-dioxolo-2,2-dimethyl-8H,9H,4,7-dimethylcyclohexa-4,6-diene (compound IB). The sample was dissolved in carbon tetrachloride, and the spectrum was recorded at 60 MHz. Tetramethylsilane was used as the internal standard.*

were unsuccessful. The reaction product from 10 liters of culture filtrate was extracted into ethyl acetate, and the solvent was removed to leave 1.10 g of a brown oil. Thin-layer chromatography of this oil in solvent A revealed the presence of a small amount of compound III and a large amount of a new product with an R_f of 0.60 (compound IIIA) in solvent A. Attempts to resolve this mixture by using a column of deactivated silica gel led to the isolation of compound IIIA, and all efforts to recover compound III from the column were unsuccessful. Compound IIIA had infrared and PMR spectra identical to those given by a synthetic sample of 2,4-dimethylphenol. It is thus reasonable to assume that the product formed from *m*-xylene by *P. putida* 39/D was 3,5-dimethyl-3,5-cyclohexadiene-1,2-diol. Since this product is very unstable it dehydrates to yield 2,4-dimethylphenol.

The parent strain of *P. putida* would not utilize either *p*- or *m*-xylene as growth substrates. However, when the organism was grown on succinate in the presence of *p*-xylene, 3,6-dimethylpyrocatechol accumulated in the culture medium. Similarly, *m*-xylene was oxidized to a catechol that was presumed to be 3,5-dimethylpyrocatechol. The latter product was not isolated.

The pathway proposed for the oxidation of *p*-xylene by *P. putida* is shown in Fig. 5. An analogous sequence is envisaged for *m*-xylene.

DISCUSSION

Figure 1 shows one of the two pathways proposed by Jamison et al. (13) for the oxidation of *p*-xylene by *N. corallina* V-49. From the results reported here, it would seem quite probable that the compound represented by X is *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (*cis*-*p*-xylene dihydrodiol). This product accumulates in the culture medium when *P. putida* 39/D is grown on succinate in the presence of *p*-xylene. The *cis* stereochemistry of the hydroxyl groups is supported by the observation that the isolated product forms an isopropylidene derivative with 2,2-dimethoxypropane (3). Further support is provided by previous investigations that have shown that *P. putida* 39/D oxidizes benzene to *cis*-3,5-cyclohexadiene-1,2-diol (9) and toluene to 3-methyl-3,5-cyclohexadiene-1S,2R-diol (15, 21). By analogy we have proposed that *P. putida* 39/D oxidizes *m*-xylene to *cis*-3,5-dimethyl-3,5-cyclohexadien-1,2-diol. However, this product was unstable and all attempts to isolate it led to the formation of 2,4-dimethylphenol.

The parent strain of *P. putida* utilizes benzene, toluene, and ethylbenzene as carbon sources for growth (12). However, the presence of two alkyl substituents on the aromatic nucleus results in the inability of the compound to serve as a growth substrate. This may be due to a decrease in the efficiency of the dioxygenases that catalyze "meta" fission of the aromatic

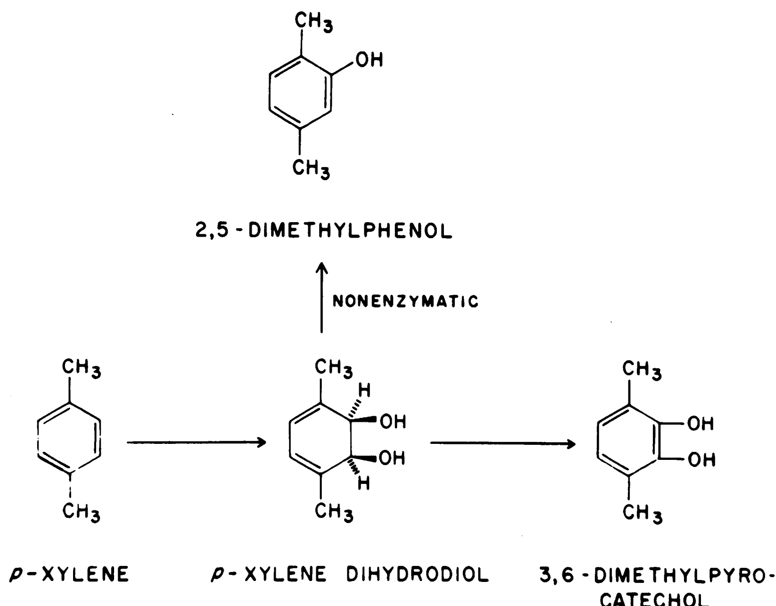

FIG. 5. *Pathway proposed for the oxidation of p-xylene by P. putida.*

nucleus. As a result, catechols and their autooxidation products accumulate in the culture medium and inhibit the growth of the organism. In support of this hypothesis we have shown that the parent strain of *P. putida*, when grown on succinate in the presence of *p*- or *m*-xylene, accumulates 3,6- and probably 3,5-dimethylpyrocatechol in the culture medium. Also, in experiments not reported here, we have demonstrated that 3-methylpyrocatechol dioxygenase, isolated from cells of *P. putida* that were grown on toluene, shows very little activity with 3,6-dimethylpyrocatechol. It is of interest to note that *N. corallina* V-49 catalyzes ring cleavage between the hydroxyl groups ("ortho" fission) of 3,6-dimethylpyrocatechol to yield α,α'-dimethyl-*cis,cis*-muconic acid (13). Those microorganisms that can utilize dimethylated aromatic hydrocarbons for growth appear to oxidize one of the methyl groups to a carboxyl group before cleavage of the aromatic nucleus (5, 6, 16).

Rat liver microsomes oxidize *p*-xylene to 3,6-dimethylphenol and *p*-toluic acid, whereas *m*-xylene is oxidized predominately to 2,4-dimethylphenol and a trace of 2,6-dimethylphenol. The origin of the apparent oxygen migration to give the latter compound is unknown (14). Interestingly, the acid-catalyzed dehydration of the dihydrodiols formed from *p*- and *m*-xylene by *P. putida* 39/D also leads to the formation of 3,6- and 2,4-dimethylphenol, respectively. However, it appears that the phenols formed by rat liver microsomes are a result of the isomerization of the corresponding dimethylated arene oxides. Mono-oxygenation of aromatic hydrocarbons by eukaryotic cells, to yield arene oxides, is a well-established phenomenon, and the isomerization of such oxides to yield phenolic metabolites occurs by a reaction that has been termed the "NIH shift" (4). In contrast, prokaryotic cells utilize a dioxygenase reaction to oxidize aromatic hydrocarbons, and *cis*-dihydrodiols are the first detectable products (8). In addition, *cis*-dihydrodiols have also been identified as intermediates in the bacterial oxidation of aromatic compounds that are not hydrocarbons. These include benzoic acid (19) and 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone (7).

It is possible that *cis* hydroxylation of substrates that do not contain a hydroxyl group is a common reaction in the bacterial oxidation of aromatic compounds.

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