# Initial Reactions in the Oxidation of Ethylbenzene by *Pseudomonas putida*†

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ABSTRACT: *Pseudomonas putida* 39/D oxidizes ethylbenzene to (+)-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol (I). Cell extracts prepared from the parent strain of *P. putida* catalyze a nicotinamide adenine dinucleotide dependent oxidation of I to 2,3-dihydroxy-1-ethylbenzene (II). In addition to I, *P. putida* 39/D also oxidizes ethylbenzene to +-*cis*-3-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III). The latter product is identical with III formed from (+)-1-phenylethanol by the same organism. Acetophenone is oxidized by *P. putida* 39/D to *cis*-3-(1′-oxoethyl)-3,5-cyclohexadiene-1,2-diol. The initial reactions utilized by *P. putida* during growth on ethylbenzene involve oxygenation to form I followed by dehydrogenation to give II. The pathway initiated by benzylic oxidation appears to be of minor importance to the organism.

The *in vivo* and *in vitro* oxidation of ethylbenzene by mammals has been studied in detail. Administration of this hydrocarbon to rabbits results in the urinary excretion of both optical isomers of 1-phenylethanol (Smith *et al.*, 1954). Rats oxidize ethylbenzene to 90.3% (*R*)-(+)-1-phenylethanol and 9.7% of the *S*-(−) isomer (McMahon and Sullivan, 1966). Studies with microsomal preparations, from rat liver, have shown that the formation of 1-phenylethanol proceeds with the incorporation of atmospheric oxygen (McMahon *et al.*, 1969). The hydroxylation reaction occurs by the direct displacement of one of the $\alpha$ hydrogens of ethylbenzene by oxygen. Oxygenation occurs with retention of configuration. Pretreatment of rats with phenobarbitol lowers the stereospecificity of the microsomal hydroxylation reaction (McMahon and Sullivan, 1966; McMahon *et al.*, 1969).

In addition to 1-phenylethanol, rats also oxidize ethylbenzene to 4-hydroxyethylbenzene (Bakke and Scheline, 1970). Rat liver microsomes produce 4-hydroxyethylbenzene and 2-hydroxyethylbenzene from the parent hydrocarbon. It seems probable that these phenolic products are formed by isomerization of the corresponding arene oxides (Kaubisch *et al.*, 1972).

At this time there have been no detailed reports on the metabolism of ethylbenzene by microorganisms that can use this hydrocarbon as sole source of carbon and energy. Davis and Raymond grew a *Nocardia* species on octadecane in the presence of ethylbenzene (Davis and Raymond, 1961). Under these conditions the aromatic hydrocarbon was converted to phenylacetic acid which was resistant to further degradation. Growth of a *Pseudomonas* species on *p*-diethylbenzene and yeast extract led to the isolation of *p*-ethylphenylacetic acid from the culture medium. Studies with deuterated *p*-diethylbenzene indicated that the major pathway of oxidation involves terminal oxidation of the alkyl side chain and not dehydrogenation followed by hydration (Tanabe *et al.*, 1971).

It is apparent that the ethylbenzene molecule possesses several different positions that are susceptible to oxidation. The site of the initial reaction varies with the source and specificity of the enzyme system involved. We now wish to report the initial reactions utilized by *Pseudomonas putida* to metabolize ethylbenzene. Oxygenation occurs at positions 2 and 3 of the aromatic nucleus in a manner analogous to that reported for toluene and its para-halogenated derivatives (Gibson *et al.*, 1968b, 1970). A minor metabolic pathway involves initial oxidation of the side chain in a manner similar to that reported for the microsomal oxidation of ethylbenzene (Smith *et al.*, 1954).

## Materials and Methods

*Analytical Methods.* Ultraviolet and visible spectra were determined on a 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 disks. Noncrystalline samples were run on neat liquid films between NaCl disks. 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 spectra were recorded on Varian A-60, HA-100, or Perkin-Elmer R-12 spectrometers. Absorptions were assigned $\delta$ values at the midpoint of half-height and are referenced to $Me_4Si$. The following abbreviations are used in pmr peak descriptions: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broadened), and sym (symmetrical). Carbon–hydrogen combustion analyses were performed by Chemalytics Inc., Tempe, Ariz. 85282. Optical rotation measurements were made on a Perkin-Elmer Model 141 polarimeter. Melting points were obtained by use of a Büchi 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) chloroform–acetone (80:20, v/v), (b) ether–hexane (50:50, v/v), and (c) benzene–acetone

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(95:5, v/v). Compounds were located on chromatograms by spraying with a 2% (w/v) solution of 2,6-dichloroquinone-4-chloroimide and also by the use of ultraviolet light. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Details of individual experiments appear in the legends to the figures.

*Organisms and Growth Conditions. P. putida* and *P. putida* 39/D were grown as previously described except that ethylbenzene replaced toluene as the oxidation substrate (Gibson *et al.*, 1970). *P. putida* 39/D is a mutant strain which oxidizes several different aromatic hydrocarbons to *cis*-diols.

*Preparation of Cell Extracts.* Cells of *P. putida* that had been grown on ethylbenzene, or cells of *P. putida* 39/D that had been grown on glucose in the presence of ethylbenzene, were washed three times with 0.02 M $KH_2PO_4$ buffer (pH 7.2). The washed cells were lyophilized and then ground, in a chilled mortar, with 0.05 M $KH_2PO_4$ buffer (pH 7.2) containing 10% ethanol (ethanol buffer). The resulting suspension was centrifuged at 26,000*g* for 1 hr. The clear supernatant solution (crude extract) was centrifuged at 105,000*g* for 1 hr. The supernatant solution ("spinco extract") was taken as a source of soluble enzymes. The spinco extract was treated with 0.2 volume of a 2% (w/v) solution of protamine sulfate. The precipitate was removed by centrifugation and the clear supernatant solution was brought to 70% saturation with finely ground ammonium sulfate. The precipitate obtained by ammonium sulfate treatment was dissolved in ethanol buffer, and dialyzed overnight against 10 l. of the same buffer. The extract prepared in this manner (ammonium sulfate extract) was dispensed into 15 ml of serum bottles, flushed with nitrogen for 15 min, and stored at −15°.

*Materials.* All chemicals were of the highest purity commercially available. Ethylbenzene was purified by distillation prior to use. Lactic acid dehydrogenase, pyruvic acid and $NAD^+$ were from Sigma. (+)- and (−)-phenylethanol were from K & K Laboratories, Inc., Plainview, N. Y. Silica gel 60 (Brinkmann Instruments Inc.) was used for column chromatography.

All other materials and methods are as previously described (Gibson *et al.*, 1970).

*Isolation of (+)-cis-3-Ethyl-3,5-cyclohexadiene-1,2-diol (I). P. putida* 39/D was grown in 10 l. of glucose (0.2%) mineral salts medium in a New Brunswick Model M14 fermentor. Ethylbenzene was supplied to the growth medium by blowing air at a rate of 2 l./min through an erlenmeyer flask that contained 100 ml of ethylbenzene. The outlet port of this flask was connected to a sparger that was placed 6 cm above the bottom of the culture vessel. Air was supplied to the culture at a rate of 10 l./min and the temperature was maintained at 30°. The contents of the fermentor were stirred at 800 rpm. Growth of the organism was monitored turbidimetrically at 600 nm. The appearance of compound I was detected by taking 1.0-ml samples at time intervals. These cell suspensions were centrifuged at 20,000*g* for 15 min. A 0.05-ml sample of the clear supernatant solution was diluted to 3.0 ml with distilled water and the absorption of this solution at 265 nm was used to calculate the amount of compound I present in the culture medium. After 8 hr the turbidity of the culture at 600 nm was 0.935 and the amount of compound I present was 0.10 mol (14.0 g). This amount was calculated using $\epsilon$ = 5783 and represents a 38% conversion of ethylbenzene (0.262 mol) into compound I. At this time the cells in the culture were removed by centrifugation. The clear supernatant solution was brought up to pH 8.0 with 5 N NaOH and extracted in 2-l. portions with 3 l. of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo* to leave 12 g of a brown oil. Five recrystallizations from hexane gave 7.4 g of pure material: mp 38°; $[\alpha]_D^{25}$ +42° (*c* = 0.035, MeOH); $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5783); $\lambda_{max}^{Nujol}$ 2.9, 6.06, 6.26, and 12.35 $\mu$. *Anal.* Calcd mass for $^{12}C_8{}^1H_{12}{}^{16}O_2$: 140.0837. Found: 140.0829.

*Acid-Catalyzed Dehydration of Compound I.* Compound I (140 mg) was dissolved in 10 ml of 3 N HCl. After 10 min at room temperature the solution was extracted with an equal volume of ether. The ether extract was dried over anhydrous sodium sulfate and the solvent was removed to leave a brown oil. Chromatography in solvent C revealed the presence of a major compound, $R_F$ 0.43; a minor compound, $R_F$ 0.25; and a trace of a more polar compound, $R_F$ 0.11. The major and minor compounds were chromatographically identical with synthetic samples of 2- and 3-ethylphenol, respectively. The brown oil was applied to a silica gel column (11 × 1 cm) and eluted with benzene; 3.0-ml fractions were collected. Fractions 7–15 were pooled; the solvent was removed to leave 81 mg of a pale yellow oil. The infrared spectrum of this compound was identical to that given by a synthetic sample of 2-ethylphenol. The oil was dissolved in 3.0 ml of anhydrous pyridine. To this solution was added 130 mg of *p*-nitrobenzoyl chloride. The reaction mixture was warmed over a low flame for 1 min and then poured into 10 ml of water. The resulting precipitate was allowed to settle and the supernatant liquid was discarded. After stirring the residue with 5 ml of 5% (w/v) $Na_2CO_3$ the suspension was filtered and the residue was recrystallized from ethanol. The *p*-nitrobenzoate derivative (mp 52–53°) did not depress the melting point of a synthetic sample prepared from 2-ethylphenol. Fractions 19–20 from the column were pooled and the solvent was removed to leave ~2 mg of a yellow oil. The infrared spectrum of this compound was identical to that given by a synthetic sample of 3-ethylphenol. The more polar compound formed during the acid-catalyzed dehydration of compound I was not present in sufficient amounts for chemical characterization.

*Acetylation of Compound I.* Compound I (300 mg) was dissolved in 3.70 ml of pyridine. Acetic anhydride (2.50 ml) was added to the reaction mixture and the resulting solution was allowed to stand at room temperature for 18 hr. The solvent was removed under vacuum to leave 439 mg of a brown oil. Silica gel column chromatography gave a pure sample of (+)-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol diacetate (IA): $[\alpha]_D^{25}$ +4° (*c* 0.059, MeOH); $\lambda_{max}^{MeOH}$ 263 nm ($\epsilon$ 6031); $\lambda_{max}^{film}$ 5.7, 6.01, 6.24, and 8.05 $\mu$. *Anal.* Calcd mass for $^{12}C_{12}{}^1H_{16}{}^{16}O_4$: 224.1048. Found: 224.1047.

*Preparation of 1-Ethyl-2,3-endo-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-endo-dicarboxylic Anhydride (IB).* Compound IA (390 mg) was dissolved in 10 ml of dry benzene that contained 200 mg of maleic anhydride. After 2 weeks at room temperature, the solvent was removed and the residue was recrystallized from acetone–hexane (yield 150 mg): mp 170–171°; $\lambda_{max}^{Nujol}$ 5.6, 5.7, and 8.04 $\mu$. *Anal.* Calcd mass for $^{12}C_{16}{}^1H_{18}{}^{16}O_7$: 322.1052. Found: 322.1053. Calcd for $C_{16}H_{18}O_7$ (322): C, 59.62; H, 5.63. Found: C, 59.73; H, 5.54.

*Enzymatic Formation of 2,3-Dihydroxy-1-ethylbenzene (II).* The reaction mixture contained in 50 ml of 0.05 M $KH_2PO_4$ buffer (pH 7.2): crude cell extract, prepared from cells of *P. putida* after growth on ethylbenzene (375 mg of protein), NAD (0.03 mmol), sodium pyruvate (2.00 mol), and lactic acid dehydrogenase (6.5 mg of protein). The above components were placed in a stoppered serum bottle which was then flushed with nitrogen for 45 min. Compound I (1.8 mmol),

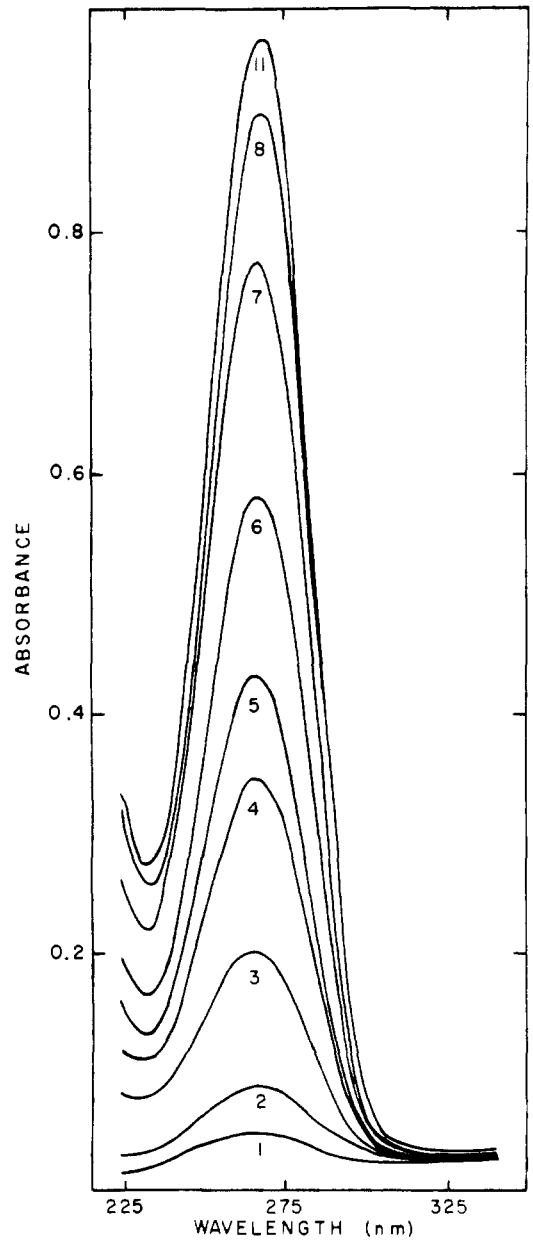



FIGURE 1: Production of compound I by *P. putida* 39/D. A 10-l. culture was grown as described in Materials and Methods. At the times indicated (hours) a sample of the culture was centrifuged to remove the cells, 0.05 ml of the clear supernatant solution was diluted to 3.0 ml with distilled water and the absorption spectrum was measured on a Cary Model 14 recording spectrophotometer.

in 10 ml of 0.05 M $KH_2PO_4$ buffer (pH 7.2), was injected through the serum cap into the reaction mixture. After 1.5 hr, 10 ml of 5 N HCl was added to stop the reaction. Precipitated protein was removed by centrifugation and the clear supernatant solution was extracted with two volumes of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and the solvent was removed to leave 297 mg of a brown oil. Silica gel column chromatography gave 140 mg of II as a pale yellow oil: $\lambda_{max}^{MeOH}$ 277 nm ($\epsilon$ 2000); $\lambda_{max}^{oil}$ 2.9, 6.7, 6.28, 6.74, and 8.4 $\mu$.

*Isolation of cis-3-(1'-Hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III).* The concentrated mother liquors remaining after crystallization of I were found to contain 107 mg of a more polar compound. After silica gel column chromatography this metabolite was obtained chromatographically pure as a colorless oil: $[\alpha]_D^{25}$ +54° (*c* 0.201, MeOH); $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5639); $\lambda_{max}^{oil}$ 2.9, 6.05, 6.25, and 7.15 $\mu$.

*Acetylation of III.* Compound III (237 mg), prepared as described above, was dissolved in 1.70 ml of pyridine. Acetic anhydride (1.2 ml) was added to the mixture and the reaction was allowed to proceed at room temperature for 2 days. The solvent was removed under vacuum to leave 399 mg of brown oil. Silica gel column chromatography gave 315 mg of *cis*-3-(1'-acetoxyethyl)-3,5-cyclohexadiene-1,2-diol diacetate (IIIA) as a colorless oil: $\lambda_{max}^{MeOH}$ 258 nm ($\epsilon$ 4130); $\lambda_{max}^{oil}$ 5.75, 6.05, 6.25, and 8.10 $\mu$; $[\alpha]^{25}$ −7° (*c* 0.066, MeOH). *Anal.* Calcd mass for $^{12}C_{14}{}^{1}H_{18}{}^{16}O_6$: 282.1096. Found: 282.1103.

*Formation of 1-(1'-Acetoxyethyl)-2,3-endo-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-endo-dicarboxylic Anhydride (IIIB).* Compound IIIA (330 mg) was dissolved in 12 ml of dry benzene which contained 420 mg of maleic anhydride. After 4 weeks the solvent was removed and the residue was shaken with 10 ml of 5% (w/v) $Na_2CO_3$. The suspension was extracted with three volumes of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and the solvent was removed to leave a solid residue (43 mg) which was crystallized from hexane: mp 205–206°; $\lambda_{max}^{Nujol}$ 5.6, 5.69, 5.75, and 8.05 $\mu$. *Anal.* Calcd mass for $^{12}C_{18}{}^{1}H_{20}{}^{16}O_9$: 380.1098. Found: 380.1107.

*Preparation of induced cells of P. putida 39/D. P. putida* 39/D was grown on glucose in the presence of ethylbenzene as described for the isolation of I. Cells were harvested in the mid log phase of growth by centrifugation. The cells were washed four times with 0.05 M $KH_2PO_4$ buffer (pH 7.2) and resuspended in the same buffer (15 mg/ml dry weight). The cell suspension was divided into five 300-ml portions and used in the following experiments.

i. OXIDATION OF ACETOPHENONE. Acetophenone (500 mg) was added to 300 ml of cell suspension in a 2-l. erlenmeyer flask. The experiment was performed in duplicate. The flasks were shaken at 30°. After 6 hr the cell suspension was centrifuged at 20,000*g* for 15 min. The supernatant solution was lyophilized and the residue extracted with 3 × 100 ml portions of ethyl acetate. The solvent was removed under vacuum and the residue was dissolved in 1.0 ml of $CHCl_3$. The chloroform solution was applied to a silica gel column (25 × 1 cm) and eluted with chloroform–acetone (1:1), and 5-ml fractions were collected. Fractions 6–11 were pooled and the solvent was removed to leave 33 mg of 3-(1'-oxoethyl)-3,5-cyclohexadiene-1,2-diol (IV): mp 119–120°; $\lambda_{max}^{MeOH}$ 295 nm ($\epsilon$ 8762); $\lambda_{max}^{Nujol}$ 2.9, 6.1, and 6.4 $\mu$; $[\alpha]_D^{25}$ +104° (*c* 0.3315, MeOH). *Anal.* Calcd mass for $^{12}C_8{}^{1}H_{10}{}^{16}O_3$: 154,0630. Found: 154.0631.

ii. OXIDATION OF (±)-1-PHENYLETHANOL. The experiment described in part i was repeated using (±)-1-phenylethanol (1.0 g) as the substrate. Silica gel column chromatography gave 148 mg of a colorless oil: $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5309); $\lambda_{max}^{oil}$ 2.9, 6.05, 6.25, and 7.15 $\mu$; $[\alpha]^{25}$ +29° (*c* 0.2971, MeOH).

iii. OXIDATION OF (+)-1-PHENYLETHANOL. The above experiment was repeated with (+)-1-phenylethanol (1.0 g) and led to the isolation of 198 mg of a colorless oil: $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5505); $\lambda_{max}^{oil}$ 2.9, 6.05, 6.25, and 7.15 $\mu$; $[\alpha]_D^{25}$ +55° (*c* 1.09, MeOH).

iv. OXIDATION OF (−)-1-PHENYLETHANOL. The above experiment was repeated with (−)-1-phenylethanol (800 mg) as substrate and led to the isolation of 72 mg of a colorless oil: $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5311); $\lambda_{max}^{oil}$ 2.9, 6.05, 6.25, and 7.15 $\mu$; $[\alpha]_D^{25}$ +6° (*c* 0.72, MeOH).

v. OXIDATION OF *cis*-3-ETHYL-3,5-CYCLOHEXADIENE-1,2-DIOL.

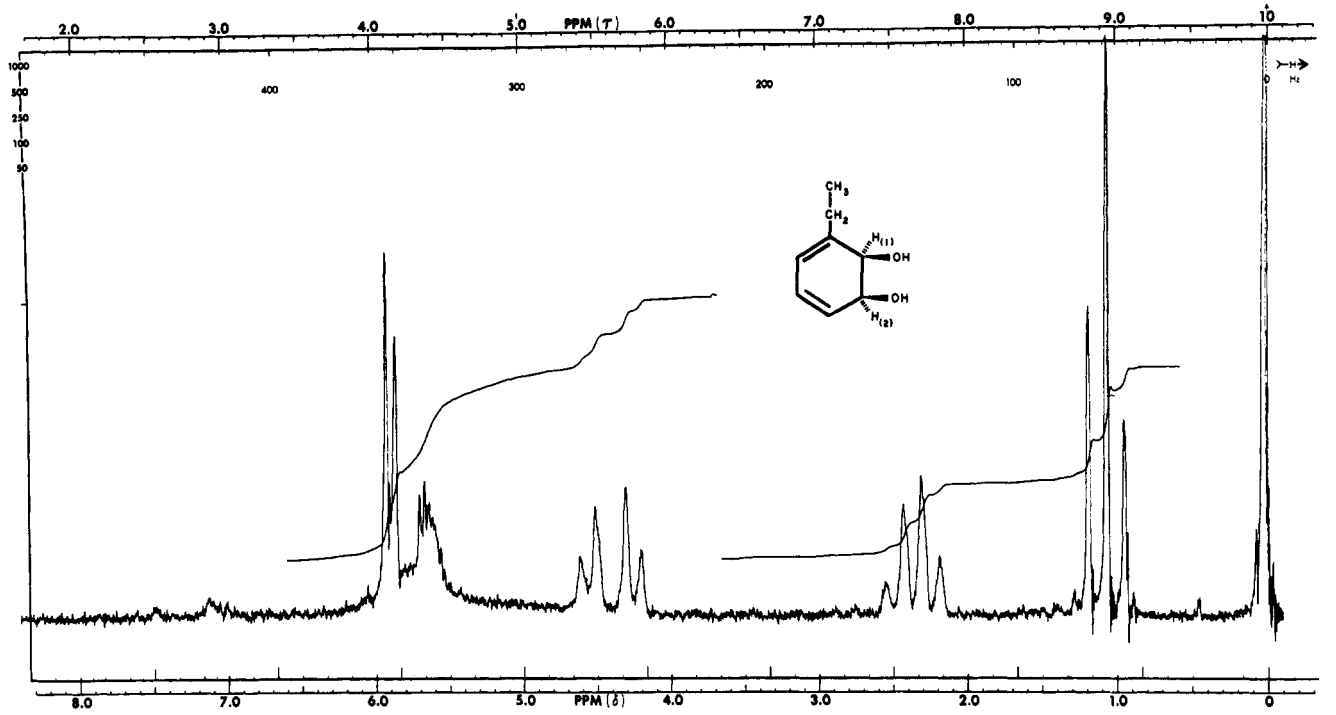


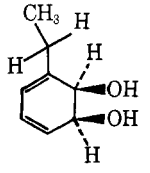

FIGURE 2: Proton magnetic resonance spectrum of *cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol (compound I). The sample was dissolved in deuterated pyridine and the spectrum recorded at 60 MHz. Tetramethylsilane was used as the internal standard.

The cell suspensions used in the above experiments did not oxidize I.

The infrared and ultraviolet spectra of III formed from each of the 1-phenylethanol samples were identical with the spectra given by III that was formed from ethylbenzene by *P. putida* 39/D.

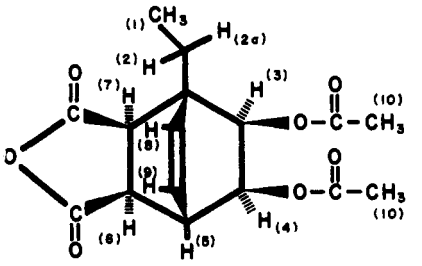
## Results and Discussion

When ethylbenzene was exposed to *P. putida* 39/D during growth on glucose a considerable amount of a neutral ultraviolet-absorbing compound (I) was excreted into the culture medium (Figure 1). Compound I was initially recognized as a substituted cyclohexadiene from its ultraviolet spectrum, $\lambda_{max}^{MeOH}$ 265 nm ($\epsilon$ 5783). Its infrared spectrum showed absorption at 2.90 $\mu$ (OH) and weak absorption at 6.06 and 6.26 $\mu$ (diene). Evidence for the adjacent positions of the hydroxyl groups was provided by acid-catalyzed dehydration of I to give 2-ethylphenol as a major product and a small amount of 3-ethylphenol. The proton magnetic resonance spectrum of I in deuterated acetone showed bands at $\delta$ 1.07, 3 H (t, methyl); $\delta$ 2.22, 2 H (q, methylene); $\delta$ 4.0, 4 H (bd OH and hydroxymethine); and $\delta$ 5.70, 3 H (m, olefinic). The above observations suggested structure I for the ethylbenzene metabolite.




3-ethyl-3,5-cyclohexadiene-1,2-diol (I)

However, the chemical shift of the hydroxyl protons and H(1) and H(2) overlapped in the pmr spectrum. This problem was resolved by running the spectrum in deuterated pyridine (Figure 2). Protons H(1) and H(2) are seen at $\delta$ 4.25 and 4.55, respectively, with coupling constants of 6.5 Hz. Acetylation of I gave 3-ethyl-3,5-cyclohexadiene-1,2-diol diacetate (IA) whose pmr spectrum in $CCl_4$ gave bands at $\delta$ 1.08, 3 H (t,




| Proton | Chemical Shift ($\delta$) | Description |
|---|---|---|
| 1 | 1.00 | 3 H (t, methyl, $J_{1,2a} = J_{1,2} = 7.4$ Hz) |
| 2 | 1.75 | 1 H (dq, methylene, $J_{2,1} = 7.4$ Hz; $J_{2,2a} = 14.8$ Hz) |
| 2a | 2.20 | 1 H (dq, methylene, $J_{2a,1} = 7.4$ Hz; $J_{2a,2} = 14.8$ Hz) |
| 3 | 4.82 | 1 H (*d*, acetoxy methine, $J_{3,4} = 8.6$ Hz) |
| 4 | 4.90 | 1 H (dd, acetoxy methine, $J_{4,3} = 8.6$ Hz; $J_{4,5} = 2.6$ Hz) |
| 5 | 3.40 | 1 H (m, bridgehead methine) |
| 6 | 3.80 | 1 H (dd, anhydric methine, $J_{6,5} = 3.1$ Hz; $J_{6,7} = 8.8$ Hz) |
| 7 | 3.60 | 1 H (d, anhydric methine, $J_{7,6} = 8.8$ Hz) |
| 8 | 6.33 | 1 H (dd, olefinic, $J_{8,9}$ Hz; $J_{8,5} = 1.0$ Hz) |
| 9 | 6.50 | 1 H (dd, olefinic, $J_{9,8} = 8.5$ Hz; $J_{9,5} = 3.6$ Hz) |
| 10 | 2.05 | 3 H (s, acetoxy methyl) |
| 10 | 2.10 | 3 H (s, acetoxy methyl) |

FIGURE 3: Analysis of the proton magnetic resonance spectrum of 1-ethyl-2,3-*endo*-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-*endo*-dicarboxylic anhydride (IB). The sample was dissolved in deuterated acetone and the spectrum was recorded at 220 MHz. Olefinic peak assignments were made by spin decoupling at H(5) on a 100-MHz instrument.[3] Tetramethylsilane was used as an internal standard.

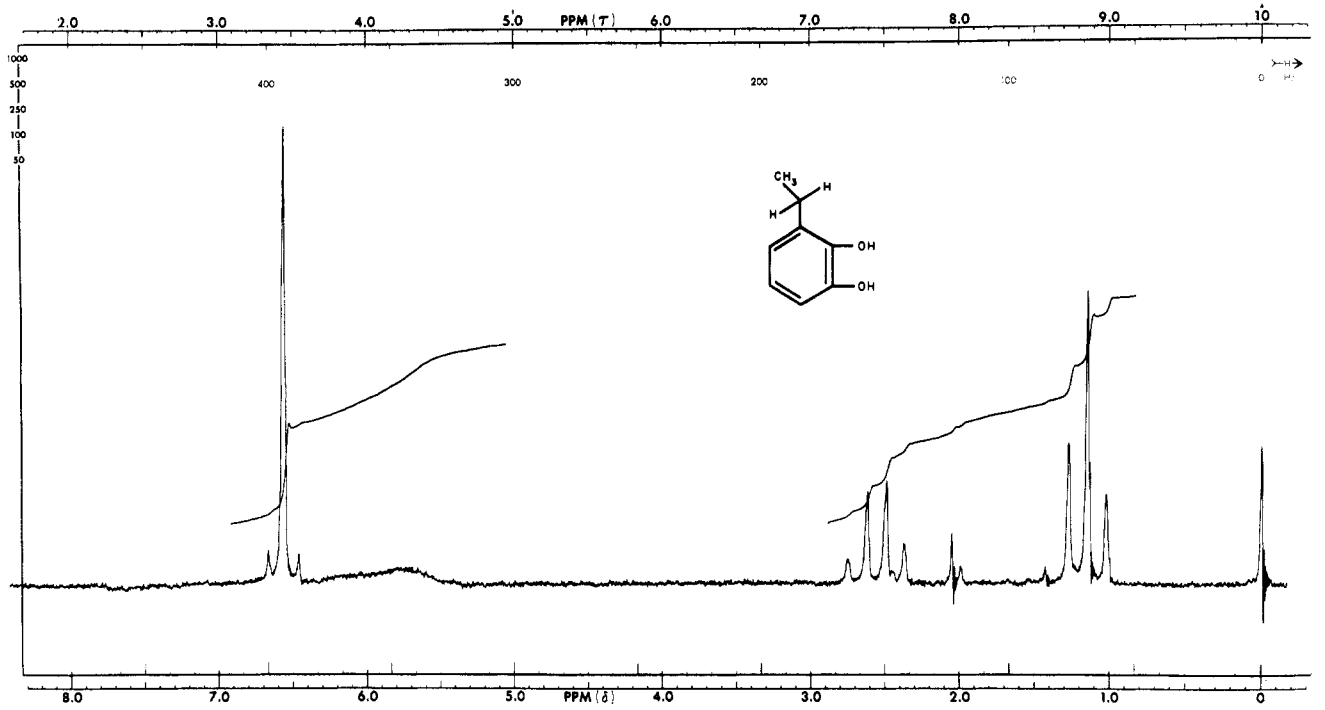



FIGURE 4: Proton magnetic resonance spectrum of 2,3-dihydroxy-1-ethylbenzene (II). The sample was dissolved in $CCl_4$ and the spectrum recorded at 60 MHz. Tetramethylsilane was used as an internal standard.

methyl); δ 1.97, 3 H (s, acetate methyl); δ 2.00, 3 H (s, acetate methyl); δ 2.12, 2 H (q, methylene); δ 5.44, 2 H (d d, acetoxymethine); and δ 5.85, 3 H (m, olefinic). Condensation of IA with maleic anhydride afforded 1-ethyl-2,3-*endo*-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-*endo*-dicarboxylic anhydride (IB).[1] Analysis of a 220-MHz pmr spectrum of IB is given in Figure 3 and shows that the structure of the compound produced from ethylbenzene by *P. putida* 39/D is (+)-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol. It should be noted that the results do not unequivocally establish the cis configuration of the hydroxyl groups in the metabolite. In a previous report we have used the Karplus relationship (Karplus, 1963) to show that *P. putida* 39/D oxidizes toluene to (+)-*cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (Gibson *et al.*, 1970). This relationship relates the coupling constants between vicinal protons to the dihedral angle between them and holds reasonably well for substituted cyclopentanes and cyclohexanes. Examination of Dreiding stereomodels of IB shows that a cis configuration of H(3) and H(4) would give a dihedral angle between these protons $\simeq 0°$. The predicted coupling constants are $\simeq 8.0$ Hz which agrees well with the experimentally observed values of 8.6 Hz. However, an insufficient number of related molecules have been examined to establish whether the Karplus relationship is applicable to the Diels–Alder adduct. The absolute stereochemistry of the diol formed from toluene by *P. putida* 39/D has been determined by X-ray analysis, by chemical degradation to (−)-(*R*)-methyladipic acid, and by a spectroscopic method.[2] The structure of the toluene diol is (+)-3-methyl-3,5-cyclohexadiene-1(*S*),2(*R*)-diol. By analogy, it is reasonable to assume that the diol formed from ethylbenzene by the same organism is (+)-3-ethyl-3,5-cyclohexadiene-1(*S*),2(*R*)-diol.

Cell extracts, prepared from *P. putida* that had been grown on ethylbenzene as sole source of carbon, oxidized I with the consumption of an equimolar amount of oxygen. Oxidation was dependent on the presence of $NAD^+$. When the oxidation was performed under anaerobic conditions in the presence of a continuous supply of $NAD^+$ a dihydroxyethylbenzene derivative was formed and subsequently isolated as an oil. The product was recognized as a hydroxylated benzene derivative from its ultraviolet spectrum, $\lambda_{max}^{MeOH}$ 277 nm (ε 2000), and its infrared spectrum which gave absorption at 2.90 (OH), 6.17, 6.28, and 6.74 μ (aromatic ring). The 60-MHz pmr spectrum in $CCl_4$ is shown in Figure 4. Signals are seen at δ 1.15, 3 H (t, methyl); δ 2.55, 2 H (q, methylene); δ 5.75, 2 H (bs, OH); and δ 6.55, 3 H (aromatic protons). These observations show that the oxidation product is 2,3-dihydroxy-1-ethylbenzene (II).

Thin-layer chromatography of the mother liquors that remained after the crystallization of I revealed the presence of a second more polar product ($R_F$ 0.15 in solvent [a]). This metabolite (III) was recognized as a hydroxylated cyclohexadiene derivative from its ultraviolet spectrum, $\lambda_{max}^{MeOH}$ 265 nm (ε 5639), and its infrared spectrum which gave absorptions at 2.90 (OH), 6.05, and 6.25 μ (diene). The compound was very unstable and readily dehydrated to yield phenolic products which were not characterized. Mass spectral analysis

[1] The endo designation for IB and IIIB refers to a syn relationship of a substituent to an unsaturated bridge and is based on the absolute stereochemical assignments made for the Diels–Alder adduct of the toluene diol.

[2] The X-ray determination was performed by R. E. Davis and A. Garza, Department of Chemistry, The University of Texas at Austin. The molecule used for X-ray analysis was the Diels–Alder adduct of *cis*-3-methyl-3,5-cyclohexadien-1,2-diol diacetate and 4-(*p*-bromophenyl)-1,2,4-triazoline-3,5-dione. Determination of absolute configuration was achieved using the methods of Bijvoet *et al.* (1951) and Hamilton (1965). The assignment of absolute stereochemistry by chemical and spectroscopic methods was performed in collaboration with Drs. H. Ziffer and D. M. Jerina, National Institutes of Health, Bethesda, Md. (to be published elsewhere).

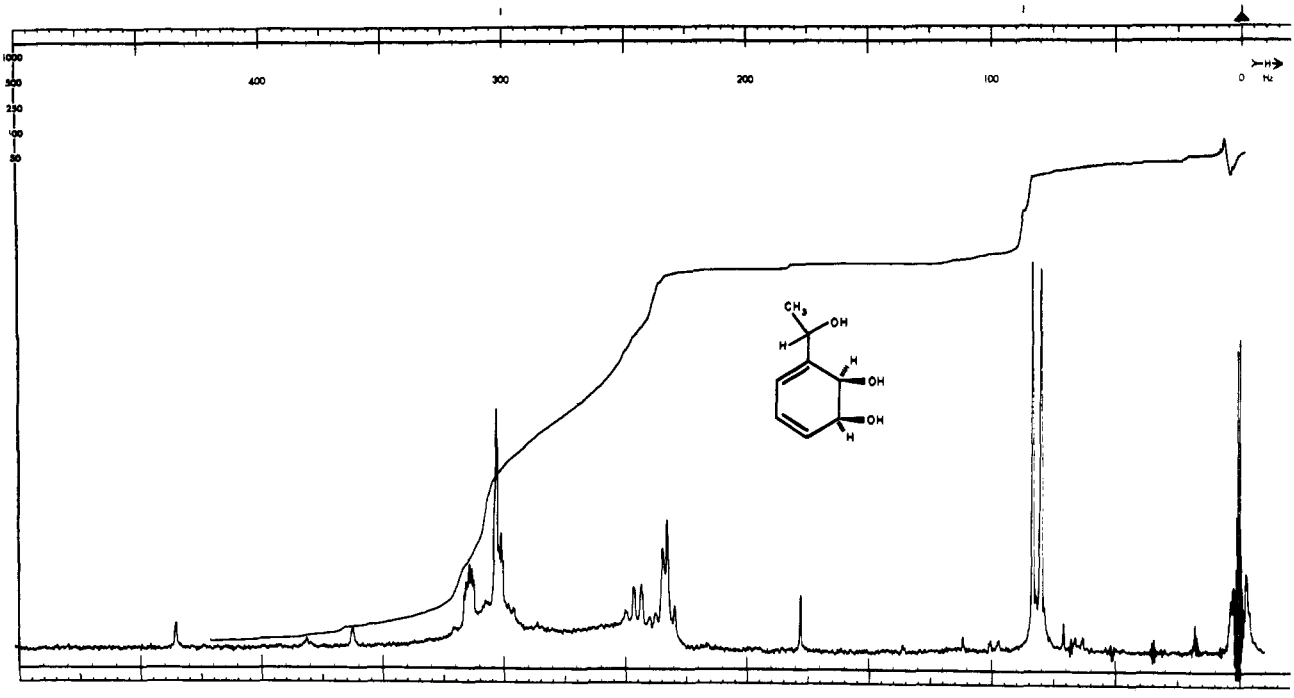
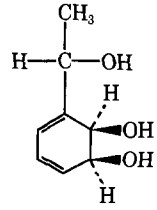


FIGURE 5: Proton magnetic resonance spectrum of *cis*-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III). The sample was dissolved in deuterated pyridine and the spectrum was recorded at 100 MHz. Tetramethylsilane was used as an internal standard.

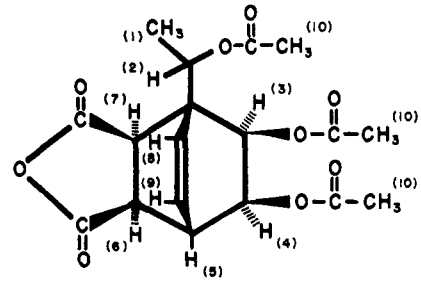
did not give a parent ion but prominent peaks were observed at *m/e* 138 ($p - H_2O$) and 120 ($p - 2H_2O$). A 100-MHz pmr spectrum is shown in Figure 5. The doublet at $\delta$ 1.64 ($J$ = 7.0 Hz) was recognized as a methyl group adjacent to a carbon carrying a methine proton. The methine absorption is seen as a quartet at $\delta$ 4.90 ($J$ = 7.0 Hz). These observations indicated that the ethylbenzene metabolite was hydroxylated at the benzylic position. Acetylation of III gave a product whose pmr spectrum in $CDCl_3$ gave bands at $\delta$ 1.30, 3 H (d, methyl); $\delta$ 2.05, 9 H (s, acetate methyl); $\delta$ 5.45, 1 H (q, acetoxy methine); and $\delta$ 6.0, 5 H (m, olefinic and acetoxy methine protons). The infrared spectrum of the acetylated product gave peaks at 5.75 (carbonyl of ester), 6.05, and 6.25 $\mu$ (diene). Mass spectral data were in accord with $C_{14}H_{18}O_5$ and in conjunction with the above results suggest that the acetylated product is 3-(1′-acetoxyethyl)-3,5-cyclohexadiene-1,2-diol diacetate (IIIA). Condensation of IIIA with maleic anhydride gave 1-(1′-acetoxyethyl)-2,3-*endo*-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-*endo*-dicarboxylic anhydride (IIIB).[1] A 100-MHz pmr spectrum of IIIB gave the results shown in Figure 6. The results given above establish that the minor product formed from ethylbenzene by *P. putida* 39/D is structure III. It should be



*cis*-3-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III)

noted that the stereochemistry of the molecule has not been rigorously established.

The identification of III introduces several possibilities as to the mechanism of its formation. These pathways are shown in Figure 7. The sequences initiated by reaction A suggests that I, which is the major product formed from ethylbenzene by *P. putida* 39/D, is the precursor of III. All of these sequences were eliminated when it was demonstrated that washed cell suspensions, that were known to be capable of converting ethylbenzene to III, failed to effect any transformation of I. Benzylic oxidation of ethylbenzene to form ethylbenzene hydroperoxide (V) could occur either enzymatically or by autoxidation. We were unable to prepare a stable sample of the hydroperoxide. However, if this compound was formed



| Proton | Chemical Shift ($\delta$) | Description |
|---|---|---|
| 1 | 1.40 | 3 H (d, methyl, $J_{1,2}$ = 7.0 Hz) |
| 2 | 5.50 | 1 H (q, acetoxy methine, $J_{2,1}$ = 7.0 Hz) |
| 3, 4 | 4.92 | 2 H (m, acetoxy methine) |
| 5 | 3.30 | 1 H (m, bridgehead methine) |
| 6, 7 | 3.80 | 2 H (m, anhydric methine) |
| 8 | 6.15 | 1 H (d, olefinic, $J_{8,9}$ = 9.0 Hz) |
| 9 | 6.38 | 1 H (dd, olefinic, $J_{9,8}$ = 9.0 Hz) |
| 10 | 2.15 | 3 H (s, acetoxy methyl) |
| 10 | 2.00 | 3 H (s, acetoxy methyl) |
| 10 | 2.03 | 3 H (s, acetoxy methyl) |

FIGURE 6: Analysis of the proton magnetic resonance spectrum of 1-(1′-acetoxyethyl)-2,3-*endo*-diacetoxybicyclo[2.2.2]oct-5-ene-7,8-*endo*-dicarboxylic anhydride (IIIB). The sample was dissolved in deuterated acetone and the spectrum recorded at 100 MHz. Tetramethylsilane was used as an internal standard.

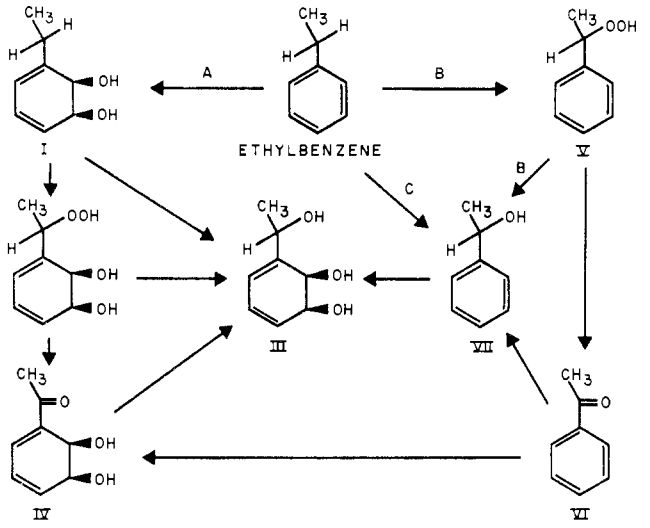



FIGURE 7: Alternative pathways for the formation of *cis*-3-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III) from ethylbenzene by *P. putida*.

it could rearrange to give acetophenone (VI) which could give III by one of the two routes shown in Figure 7. Washed cell suspensions of *P. putida* 39/D oxidized acetophenone to a compound whose properties were consistent with the structure 3-(1′-oxoethyl)-3,5-cyclohexadiene-1,2-diol (IV). There was no evidence for the formation of III in this experiment. The above experiments indicated that 1-phenylethanol (VII) was a possible precursor of III. Washed cell suspensions of *P. putida* 39/D oxidized (±)-1-phenylethanol, (+)-1-phenylethanol, and (−)-1-phenylethanol to III. The infrared and ultraviolet spectra of III formed from each of the 1-phenylethanol samples were identical with the spectra given by III that was formed from ethylbenzene by *P. putida* 39/D. However, only the optical activity of III formed from (+)-1-phenylethanol was the same as that given by III from ethylbenzene (Table I). It appears that *P. putida* 39/D stereospecifically catalyzes benzylic hydroxylation of ethylbenzene to give (+)-1-phenylethanol (VII) which is further oxidized to III (pathway C). At this time we cannot eliminate the intermediacy of ethylbenzene hydroperoxide (V) in the conversion of ethylbenzene to (+)-1-phenylethanol (pathway B). We have been unable to detect (+)-1-phenylethanol in our experiments, presumably because the side chain hydroxylation is the rate-limiting step in the conversion of ethylbenzene to III. Approximate calculations reveal that III comprises less than 2% of the total oxidation products formed from ethylbenzene.

TABLE I: Optical Activity of *cis*-3-(1′-Hydroxyethyl)-3,5-cyclohexadiene-1,2-diol (III) Formed from Different Substrates.[a]

| Source of III | $[\alpha]^{25}D$ (deg) |
|---|---|
| Ethylbenzene | +54.1 |
| (±)-1-Phenylethanol | +29.0 |
| (+)-1-Phenylethanol | +54.7 |
| (−)-1-Phenylethanol | +5.8 |

[a] Samples were dissolved in methanol and measurements determined on a Perkin-Elmer Model 141 polarimeter.

TABLE II: Oxidation of Aromatic Hydrocarbons by Cell Extracts of *P. putida* and *P. putida* 39/D.[a]

| | Specific Activity[b] | | |
|---|---|---|---|
| | *P. putida* Grown on | | *P. putida* 39/D Grown on |
| Substrate | Ethylbenzene | Ethylbenzene + Glucose | Ethylbenzene + Glucose |
| Ethylbenzene | 16.2 | 2.6 | 3.4 |
| Toluene | 19.3 | 2.7 | 3.8 |
| Benzene | 10.1 | 1.7 | 2.7 |
| Propylbenzene | 13.9 | 2.5 | 3.3 |
| Butylbenzene | 11.0 | 2.1 | 3.1 |
| *p*-Xylene | 7.7 | 1.4 | 2.1 |
| *p*-Ethyltoluene | 2.8 | 0.9 | 1.8 |

[a] Reaction mixtures contained in a final volume of 2.0 ml: $KH_2PO_4$ buffer, pH 7.2, 75 μmol; $FeSO_4 \cdot 7H_2O$, 0.2 μmol; $NAD^+$, 0.8 μmol; ammonium sulfate extract, 16 mg of protein; and substrate 0.2 μmol in 10 μl of 95% ethanol. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Results are corrected for endogenous absorption in the absence of substrate. [b] nmol of $O_2$/min per mg of protein.

It is thus unlikely that benzylic hydroxylation plays a significant part in the utilization of ethylbenzene as a source of carbon and energy for *P. putida*.

The specificity of the ethylbenzene oxygenase for different aromatic hydrocarbon substrates is given in Table II. Cell extracts prepared from *P. putida*, after growth on ethylbenzene, oxidized toluene at a faster rate than ethylbenzene. The rate of oxidation of the different substrates decreases with increasing length of the alkyl side chain. When *P. putida* is grown on toluene the same oxidation pattern is observed. The presence of glucose in the medium repressed the levels of the enzyme activity in *P. putida* by 80–85%. When *P. putida* and *P. putida* 39/D were grown on glucose, in the presence of ethylbenzene, the enzyme levels in the mutant organisms were 13–16% higher than those observed in the parent strain. However, the comparison may not be strictly valid since oxygen consumption by *P. putida* is a composite of the hydroxylation and ring fission reactions. Cell extracts from both organisms, after growth with glucose as sole source of carbon, did not oxidize the substrates listed in Table II.

The ability of cell extracts, prepared from *P. putida* after growth on ethylbenzene as sole source of carbon, to oxidize suspected intermediates in the degradation of ethylbenzene is given in Table III. (+)-1-Phenylethanol was oxidized at only 20% of the rate observed with ethylbenzene. Compounds I and II were oxidized at almost the same rate. There was no visible color change in the reaction mixture during the period of the experiment. However, compounds III formed from ethylbenzene and (+)-1-phenylethanol, respectively, were oxidized to an acid that gave a bright yellow color in alkaline solution. The absorption spectrum of the acid at pH 12.0 and 1.0 showed absorption maxima at 405 and 305 nm, respectively. The changes in absorption at different pH values

TABLE III: Oxidation of Ethylbenzene Metabolites by Cell Extracts.[a]

| Substrate | Sp. Act.[b] |
|---|---|
| Ethylbenzene[c] | 11.4 |
| (+)-1-Phenylethanol[c] | 2.3 |
| Compound I | 52.0 |
| Compound II | 59.3 |
| Compound III | 34.6 |
| Compound III[d] | 34.3 |

[a] Reaction mixtures contained in a final volume of 2.0 ml: $KH_2PO_4$ buffer (pH 7.2), 75 μmol; $FeSO_4 \cdot 7H_2O$, 0.2 μmol; $NAD^+$, 0.8 μmol; ammonium sulfate extract, 16 mg of protein; and substrate 0.2 μmol in 10 μl of 0.5 M $KH_2PO_4$ buffer (pH 7.2). Oxygen consumption was measured polarographically with a Clark oxygen electrode. [b] nmol of $O_2$/min per mg of protein. [c] Substrate 0.2 μmol in 10 μl of 95% ethanol. [d] Compound III formed from (+)-1-phenylethanol by *P. putida* 39/D.

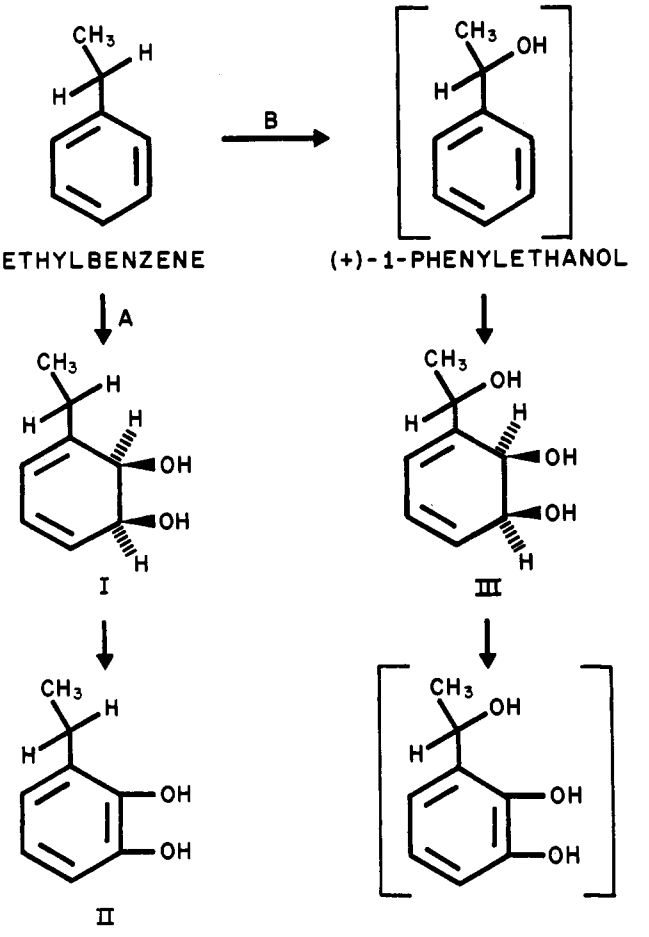



FIGURE 8: Reaction sequences utilized by *P. putida* to oxidize ethylbenzene. Compounds I, II, and III were isolated and characterized. Compounds in brackets were not isolated.

are characteristic of meta cleavage of *o*-dihydroxy aromatic compounds (Dagley *et al.*, 1964). When the oxidations of compounds I and III were performed under anaerobic conditions in the presence of $NAD^+$ a stoichiometric production of $NADH_2$ was observed for each substrate. Chromatographic examination of each reaction mixture revealed that compound I was converted to 2,3-dihydroxy-1-ethylbenzene. Each sample of compound III was also converted to a catechol derivative which was not characterized. By analogy with the product formed from compound I the structure shown in Figure 8 is proposed for the dehydrogenation product of compound III.

The data obtained indicate that *P. putida* oxidizes ethylbenzene principally by pathway A in Figure 8. Reaction sequence B is thought to be of minor importance to the organism. It seems probable that all of the reactions in sequence B, except the benzylic oxidation, are carried out by the same enzymes that are responsible for sequence A. However, it should be noted that a crude enzyme preparation from *P. fluorescens* oxidizes benzoic acid to catechol and also oxidizes 2-methylbenzoic acid to *o*-hydroxymethylbenzoic acid (Ichihara *et al.*, 1962). In view of the fact that benzoic acid is oxidized to catechol through *cis*-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (Reiner and Hegeman, 1971) it is conceivable that the enzyme(s) responsible for the oxidation of the aromatic nucleus may also be able to oxidize adjacent methyl or methylene groups. Support for this hypothesis is provided by the demonstration that a purified preparation of phenylalanine hydroxylase catalyzed the formation of a small amount of *p*-hydroxymethylphenylalanine from *p*-methylphenylalanine (Daly and Guroff, 1968).

The initial reactions in the oxidation of ethylbenzene by *P. putida* are analogous to the reactions observed in the microbial oxidation of benzene (Gibson *et al.*, 1968a), toluene (Gibson *et al.*, 1970), *p*-chlorotoluene (Gibson *et al.*, 1968b), and naphthalene (Jerina *et al.*, 1971). Unpublished work from our laboratory has also established similar sequences for the oxidation of *p*-fluorotoluene, *p*-bromotoluene, chlorobenzene, and biphenyl. It appears that cis hydroxylation of the aromatic nucleus may be a common reaction utilized by bacteria for the oxidation of aromatic hydrocarbons (Gibson, 1971).

[3] We are indebted to Dr. D. M. Jerina for obtaining and interpreting the 220-MHz pmr spectrum.

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# N-Terminal Sequence of the Eucaryotic *in Vitro* Product Made upon Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid†

William H. Klein and John M. Clark, Jr.*

ABSTRACT: This paper presents a thorough analysis of the N-terminal amino acid sequence of the satellite tobacco necrosis virus (STNV) coat protein produced by translation of STNV ribonucleic acid in an *in vitro* wheat embryo system. Short term incubations detect a limited incorporation of [$^{35}$S]methionine from [$^{35}$S]Met-tRNA$_i^{Met}$. This incorporated [$^{35}$S]Met is largely lost on prolonged incorporation. Sucrose gradient analysis of protein synthesis initiation complexes of [$^{3}$H]Met-tRNA$_i^{Met}$, STNV-RNA, and wheat embryo ribosomes demonstrate that formation of the complexes requires the methionine-specific initiator tRNA. Electrophoretic resolution of labeled products found in these complexes detects the STNV-RNA dependent production of the peptide Met-Ala-Lys and its precursors. Comparison of this apparent N-terminal sequence with the Ala-Lys · · · N-terminal sequence of *in vivo* STNV coat protein dictates that STNV-RNA translation by an *in vitro* procaryotic system in that correct translation begins on a Met codon immediately prior to the codons governing the final *in vivo* sequence.

Many reports describe the *in vitro* translation of a messenger or viral RNA in heterologous cell-free extracts (Heywood, 1969; Housman *et al.*, 1971; Lane *et al.*, 1971; Laycock and Hunt, 1969; Stavnezer and Huang, 1971). Proof of the accuracy of such heterologous *in vitro* translation requires characterization of the *in vitro* products. In particular, proof of common initiation signals between mRNAs of various origins requires N-terminal amino acid sequence analysis of the *in vitro* products.

We have previously reported the *in vitro* translation of STNV-RNA[1] in both procaryotic (*Escherichia coli*) and eucaryotic (wheat embryo) extracts produces protein containing the amino acid sequence of STNV coat protein (Klein *et al.*, 1972). STNV coat protein (*in vivo*) has the N-terminal sequence I

$$\text{Ala-Lys-}\begin{matrix}\text{Glu}\\\text{Gln}\end{matrix}\cdots \qquad \text{I}$$

(Klein *et al.*, 1972). The major *in vitro* product of procaryotic translation of STNV-RNA has the N-terminal sequence fMet-Ala-Lys · · · (Lundquist and Clark, 1971; Lundquist *et al.*, 1972). This suggests that procaryotic translation of STNV-RNA initiates with an fMet immediately prior to the eventual *in vivo* N terminus. The *in vitro* eucaryotic translation of STNV-RNA yields a product containing alanine as the predominant N-terminal amino acid (Lundquist *et al.*, 1972). This paper reports that this N-terminal alanine arises from a eucaryotic initiation of STNV-RNA translation with the sequence Met-Ala-Lys · · · followed by specific removal of the N-terminal methionine. Thus both the procaryotic and eucaryotic *in vitro* systems initiate STNV-RNA translation at the same site.

## Experimental Procedures

*Materials.* STNV and STNV-RNA were prepared as described previously (Liu *et al.*, 1969; Clark and Klein, 1973). The cell-free wheat embryo system used was an S-23 extract (Marcus *et al.*, 1968) or ribosomes and S-100 derived from such S-23 extracts. Wheat embryo ribosomes were washed two times (Marcus, 1970). S-100 extracts were further freed of endogenous tRNA by passage over DEAE-cellulose preequilibrated with 0.1 M Tris-Cl, pH 7.4, and 8 mM $\beta$-mercaptoethanol. The resultant DEAE S-100 was then equilibrated with 1 mM Tris-Cl, pH 7.4, 2 mM $Mg(OAc)_2$, 50 mM KCl, and 5 mM $\beta$-mercaptoethanol, by brief (2 hr) dialysis.

Crude tRNA was prepared from wheat germ (Glitz and Dekker, 1963). The two methionine-accepting tRNAs (tRNA$_i^{Met}$ and RNA$_M^{Met}$) of this preparation were resolved from each other on benzoylated DEAE-cellulose (Leis and Keller, 1970) and then separately aminoacylated with [$^{35}$S]Met according to the method of Loehr and Keller (1968).

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received November 17, 1972.* Supported in part by National Institutes of Health Research Grant No. GM-08647. Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid. III. See Lundquist *et al.* (1972) for the preceding paper in the series.

[1] Abbreviations used are: STNV, satellite tobacco necrosis virus; tRNA$_i^{Met}$, methionine-specific initiator tRNA not capable of accepting a formyl group on its methionine; Met-tRNA$_i^{Met}$, methionyl ester containing tRNA$_i^{Met}$; tRNA$_M^{Met}$, noninitiator methionine-specific tRNA; Met-tRNA$_M^{Met}$, methionyl ester containing tRNA$_M^{Met}$.