Oxidative Degradation of Aromatic Hydrocarbons by Microorganisms. I. Enzymatic Formation of Catechol from Benzene*

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ABSTRACT: Washed cell suspensions of *Pseudomonas putida*, grown with toluene as sole source of carbon, oxidized benzene, toluene, and ethylbenzene, at equal rates. Cell extracts were used to study benzene degradation and were shown to require nicotinamide–adenine dinucleotide, ethanol, ferrous ions, and L-cysteine for maximal activity. The benzene oxygenase system consists of at least two protein fractions. Catechol and *cis*-benzene glycol have been implicated as intermediates in benzene degradation and cell extracts have been shown to contain a specific dehydrogenase for the latter compound. A reaction sequence for the enzymatic formation of catechol from benzene is proposed.

Catechol has been implicated as an intermediate in the microbial degradation of benzene on the basis of sequential induction experiments (Kleinzeller and Fencl, 1952; Wieland *et al.*, 1958; Marr and Stone, 1961). Further evidence was supplied by Marr and Stone (1961) who detected catechol chromatographically in the culture medium of *Pseudomonas aeruginosa* growing with benzene as the sole source of carbon.

The role of phenol in benzene degradation is uncertain, and evidence for, or against, this compound as an intermediate product in benzene metabolism is based solely on sequential induction experiments (Kleinzeller and Fencl, 1952; Wieland *et al.*, 1958; Marr and Stone, 1961).

Details of benzene degradation by cell extracts are scarce (Treccani and Bianchi, 1959) and consequently little is known about the intermediates involved in the early stages of benzene oxidation. By analogy with the bacterial oxidation of naphthalene (Walker and Wiltshire, 1953), Marr and Stone (1961) postulated *trans*-benzene glycol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) to be an intermediate in the conversion of benzene into catechol. An enzyme, catalyzing the dehydrogenation of this compound to catechol, has been isolated from rabbit liver (Ayengar *et al.*, 1959). A similar enzyme has also been demonstrated in cells of *Pseudomonas fluorescens* grown with DL-tryptophan (Taniuchi *et al.*, 1964), although both *cis*- and *trans*-benzene glycol were eliminated as possible intermediates in the enzymatic conversion of anthranilic acid into catechol.

The identification of D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene in naphthalene metabolism (Walker and Wiltshire, 1953) and the enzymatic formation of 7,8-dihydro-7,8-dihydroxykynurenic acid from kynurenic acid (Taniuchi and Hayaishi, 1963) suggested that these compounds were formed from epoxide precursors. Booth *et al.* (1960) have proposed that an epoxide of naphthalene is formed when naphthalene is incubated with rat liver microsomes, NADPH,[1] and oxygen. Recent experiments with $^{18}O_2$ have demonstrated that only one atom of the oxygen molecule is incorporated into 1,2-dihydro-1,2-dihydroxynaphthalene (Holtzman *et al.*, 1967). These experiments, which were performed with mouse liver microsomes, provide strong evidence that 1,2-dihydro-1,2-epoxynaphthalene is one of the first products in naphthalene oxidation. Benzene epoxide (1,2-epoxycyclohexa-3,5-diene) has been postulated as a precursor of *trans*-benzene glycol in the degradation of benzene by rat liver (Sato *et al.*, 1963; Booth *et al.*, 1960).

Pathways proposed for the formation of catechol from benzene are illustrated in Figure 1 (McKenna and Kallio, 1965). Sequence 1 involves epoxidation of the benzene nucleus followed by hydrolysis of the epoxide to give *trans*-benzene glycol which then undergoes dehydrogenation to catechol. Reaction 2 depicts a monohydroxylation reaction to form phenol followed by a second hydroxylation step forming catechol. The third reaction sequence proposes an initial formation of a hydroperoxide which undergoes further hydroxylation to form catechol. At present no evidence is available to support the validity of the proposed reaction sequences.

In this communication we present evidence for intermediates involved in the early stages of benzene oxidation by a *Pseudomonas* species.

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

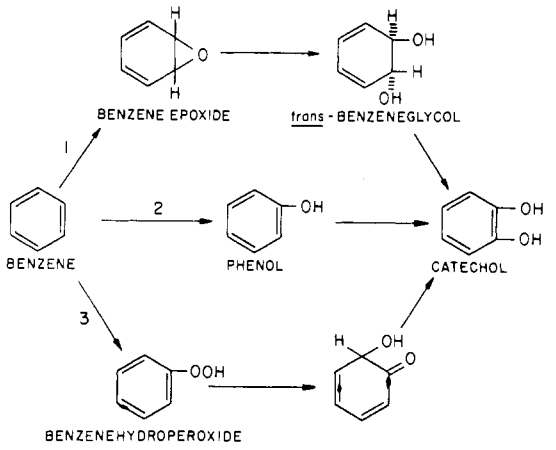

FIGURE 1: Proposed pathways for the formation of catechol from benzene.

Materials and Methods

Organism and Growth Conditions. A fluorescent pseudomonad was isolated from soil by elective culture with ethylbenzene as the carbon source. The organism has been identified as *Pseudomonas putida* biotype B (Stanier *et al.*, 1966) on the basis of gelatin liquefaction (−), egg yolk test (−), denitrification (−), growth at 40° (−), and growth on L-tryptophan (+), L-kynurenine (+), and galactose (+). Stock cultures were maintained on nutrient agar slants and subcultured every 2 weeks. Prior to inoculation into liquid medium, the organism was transferred to a mineral agar slant of the following composition in grams per liter: KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; and Bactoagar, 20. This medium was completed by the addition of 10 ml of mineral solution (Wolin *et al.*, 1963). Ethylbenzene was supplied as the carbon source by placing 0.5 ml at the bottom of the slope. The organism thus grew on the vapor without ever having direct contact with the hydrocarbon. After 24-hr growth on the aromatic hydrocarbon, the cells were used to inoculate 50 ml of liquid medium. This procedure eliminated the lag phase which sometimes occurred when cells from nutrient slants were used to inoculate liquid medium.

P. putida was grown in liquid culture identical with that described above for the preparation of mineral agar slants. The medium was supplemented with 0.1 g/l. of yeast extract. Preliminary observations revealed that the organism grew equally well with either ethylbenzene or toluene as the source of aromatic hydrocarbon, and the latter compound was used routinely as a growth substrate. Toluene was introduced to the medium in the manner described by Claus and Walker (1964). After 12-hr growth, cells were harvested in a Sharples air-driven centrifuge. The cell paste was washed with 0.02 M phosphate buffer (pH 7.2) and lyophilized. For whole cell experiments, 1 g (wet weight) of washed cells was resuspended in 20 ml of 0.02 M phosphate buffer (pH 7.2).

Preparation of Cell Extracts. Lyophilized cells (1 g) were ground in a cold mortar with 20 ml of 0.02 M phosphate buffer (pH 7.2). The resulting viscous mixture was centrifuged at 26,000*g* for 45 min, and the clear supernatant solution was used as a source of crude cell extract. Such preparation usually contained 10–15 mg of protein/ml. Protein was determined by the method of Gornall *et al.* (1949).

Partial Purification of Cell Extracts. Crude cell extracts were made 0.01 M with respect to cysteine and treated with two-tenths volumes of 2% (w/v) protamine sulfate. The resulting mixture was stirred at 0° for 10 min and then centrifuged at 0° and 20,000*g* for 15 min. The clear supernatant solution was fractionated with solid ammonium sulfate. The precipitate obtained between 30 and 70% saturation was dissolved in 0.02 M KH_2PO_4 (pH 7.2) containing 0.01 M cysteine and desalted by passing through a Sephadex G-25 column (2 × 25 cm). Elution was carried out using the same buffer.

Analytical Methods. Oxygen consumption was used as a measure of benzene degradation. Experiments were performed using conventional manometric techniques. A Cary Model 14 recording spectrophotometer was used for spectrophotometric examinations. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer with a scintillation fluid composed of 0.1 g of 1,4-bis[2-(4 methyl-5-phenyloxazolyl)]benzene and 4.0 g of 2,5-diphenyloxazole per l. of toluene. Infrared spectra were obtained with a Perkin-Elmer 137 spectrophotometer. Samples were mulled in Nujol and placed between NaCl disks. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Thin-layer chromatography was performed using Eastman Chromatogram Sheets, type K/30R (silica gel with fluorescent indicator). Solvents used for chromatography were: (a) benzene–dioxane–acetic acid (45:12.5:2.5, v/v), (b) benzene–methanol–acetic acid (90:16:8, v/v), and (c) benzene–methanol (95:5, v/v). Compounds concerned in benzene degradation were visualized under ultraviolet light and by spraying with a 2% solution of 2,6-dichloroquinone-4-chloroimide in ethanol. Catechol was determined by the method of Arnow (1937).

Materials. Kodak No-Screen Medical X-Ray film was from Eastman Kodak Co., Rochester, N. Y. Silica gel (0.05–0.20 mm) (E. Merck AG., Darmstadt, Germany) was used for column chromatography. Sephadex G-25 (100–250 mesh) was purchased from Pharmacia Fine Chemicals, Inc., New York, N. Y. Cellex D (DEAE-cellulose) and Cellex T (TEAE-cellulose) were treated as described by Guest *et al.* (1964) and were obtained from Bio-Rad, New York, N. Y. Hydroxylapatite (exchange capacity 65 mg/g) was purchased from Clarkson Chemical Corp., Williamsport, Pa. [^{14}C]Benzene (uniformly labeled) was from Nuclear-Chicago Corp., Des Plaines, Ill.

The following compounds were obtained from Sigma Chemical Co., St. Louis, Mo.: NAD^+, $NADP^+$, NADH, NADPH, FMN, FAD, alcohol dehydrogenase, glucose 6-phosphate dehydrogenase, and glucose 6-phosphate. We thank Dr. J. M. Wood for a gift of freshly prepared tetrahydrofolate, and Dr. I. C. Gunsalus for a sample of putidaredoxin. All hydrocarbons (Research

Grade) were from Phillips Petroleum Co., Bartlesville, Okla., and were redistilled prior to use. Infrared spectroscopy failed to reveal the presence of any oxygenated compounds. Catechol, 3-methycatechol, and 4-methylcatechol were from Aldrich Chemical Co., Milwaukee, Wis., and were purified by sublimation. The method of Nakajima *et al.* (1956, 1959) was used for the synthesis of *cis*- and *trans*-benzene glycol. We are indebted to Drs. M. Nakajima and N. Kurihara for gifts of *cis*- and *trans*-1,2-dihydroxy-3,4,5,6,-tetrachlorocyclohexane. All other chemicals were of the highest purity commercially available.

Results

Oxidation of Aromatic Hydrocarbons by Whole Cells. Washed cell suspensions of *P. putida*, grown with toluene as the source of carbon, oxidized benzene, toluene, and ethylbenzene at approximately equal rates; isopropylbenzene was oxidized at approximately half the rate observed with toluene, while propylbenzene and butylbenzene were only slowly metabolized (Figure 2). Pentylbenzene, hexylbenzene, heptylbenzene, octylbenzene, decylbenzene, naphthalene, anthracene, and phenanthrene were not oxidized. During the oxidation of benzene, ethylbenzene, propylbenzene, isopropylbenzene, and butylbenzene, a bright yellow color was observed in the respective reaction mixtures. The absorption maxima of these intermediates in acid and alkaline conditions are given in Table I.

TABLE I: Spectral Characteristics of the Yellow Intermediates Produced from Aromatic Hydrocarbons.[a]

Substrate	λ_{max} Yellow Intermediate (mμ)	
	pH 1.0	pH 12.0
Benzene	317	375
Ethylbenzene	319	395
Propylbenzene	319	393
Isopropylbenzene	317	392
Butylbenzene	317	392

[a] The flask contents from the experiment in Figure 2 were centrifuged and 0.5 ml of the resulting clear supernatant solutions were diluted to 3.0 ml and used for absorption studies at the stated pH values. Measurements were made against a reference cuvet which contained 0.5 ml of the supernatant solution from the endogenous control flask diluted to 3.0 ml.

When washed cell suspensions were incubated with compounds suspected of being intermediates in the metabolism of benzene and toluene the results illustrated in Table II were obtained. Toluene, benzene, catechol, 3-methylcatechol, and *cis*-benzene glycol were oxidized at approximately equal rates; phenol, *o*-cresol,

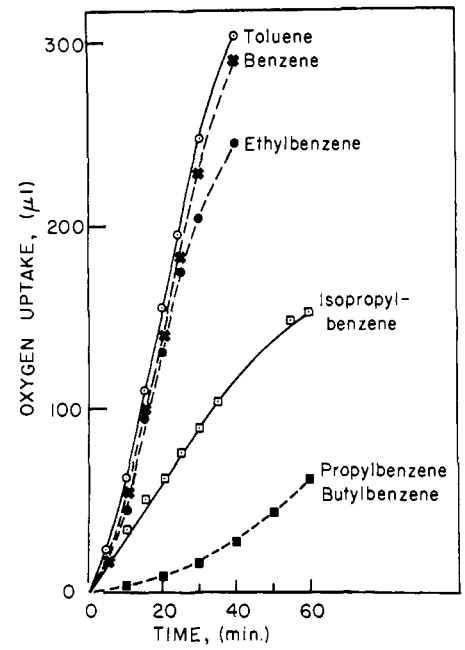

FIGURE 2: Oxidation of aromatic hydrocarbons by washed cell suspensions of *P. putida*. Warburg flasks contained, in a final volume of 3 ml: KH_2PO_4 buffer, 160 μmoles; cell suspension, 1.0 ml; and substrate, 5 μmoles in 0.2 ml of *N,N*-dimethylformamide. Results are corrected for endogenous respiration in the absence of substrate.

m-cresol, *p*-cresol, 4-methylcatechol, and *trans*-benzene glycol were metabolized at an appreciably reduced rate, while benzoic acid was not metabolized at all. When the organism was grown with yeast extract as

TABLE II: Substrate Specificity of Washed Cell Suspensions of *P. putida*.[a]

Substrates	μl of O_2 Consumed/10 min	
	Toluene Cells	Yeast Extract Cells
Toluene	93	0
Benzene	83	0
Catechol	60	10
cis-Benzene glycol	80	2
trans-Benzene glycol	5	2
Phenol	30	0
3-Methylcatechol	61	6
4-Methylcatechol	43	2
o-Cresol	21	0
m-Cresol	17	0
p-Cresol	9	0
Phenylacetic acid	0	0
Benzoic acid	0	0

[a] Conditions as described in Figure 2.

TABLE III: Substrate Specificity of Crude Cell Extract.[a]

Substrate	μl of O_2 Consumed/10 min
Benzene	74
Toluene	69
Ethylbenzene	70
Propylbenzene	48
Benzyl alcohol	62
o-Cresol	21
m-Cresol	17
p-Cresol	9
Phenol	20
3-Methylcatechol	55
4-Methylcatechol	42
3-Isopropylcatechol	41
Catechol	76
cis-Benzene glycol	63
trans-Benzene glycol	8
Benzoic acid	0
Hexadecane	0

[a] Warburg flasks contained the following in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 140 μmoles; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; and cell extract, 21 mg of protein. Substrate, 5 μmoles in 0.2 ml of 25% (v/v) ethanol, was added from the side arm. Results are corrected for endogenous respiration in the absence of substrate.

TABLE IV: Effect of Sulfydryl Compounds on Benzene Oxidation.[a]

Extract	Sulfydryl Compd (2 mM)	Act. (μl of O_2/10 min per mg of protein)
Crude	None	3.66
Fractionated	None	0.50
Fractionated	Cysteine	3.08
Fractionated	Dithioerythritol	2.00
Fractionated	Mercaptoethanol	1.91
Fractionated	Reduced glutathione	1.75

[a] Warburg flasks contained in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 140 μmoles; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; sulfydryl compound where indicated, 6.0 μmoles; and crude cell extract, 12 mg of protein, or 30–70% $(NH_4)_2SO_4$ fractionated extract, 12 mg of protein. Benzene, 2.25 μmoles in 0.1 ml of 25% (v/v) ethanol, was added from the side arm.

the source of carbon, very little oxidation of the above substrates was observed. The reaction mixtures containing benzene, *cis*-benzene glycol, and catechol were all colored deep yellow. Spectrophotometric examination of the reaction products at acid and alkaline pH revealed that the same product was produced from each substrate. The absorption maxima, 375 (pH 12) and 317 mμ (pH 2), are identical with those reported for 2-hydroxymuconic semialdehyde (Dagley *et al.*, 1960) and indicates that the organism possesses the enzymes required to convert benzene into catechol.

The failure of washed cell suspensions to oxidize benzoic acid would seem to exclude the possibility that benzene is an intermediate in toluene degradation. This was confirmed by examining culture filtrates of the toluene-grown organism for reaction intermediates. Samples of a 10-l. culture were examined at intervals for *o*-dihydroxyphenols. The presence of a catechol was detected in the early stages of growth and reached a maximum after a period of 6 hr. At this time, the cells were removed by centrifuging and the clear supernatant solution was extracted with 4 l. of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed to leave a brown residue (350 mg). The residue was applied to the top of a silica gel column (30 × 1 cm) and elution effected with benzene containing 0.5% methanol; 10-ml fractions were collected. Fractions 30–42 contained the catechol (39 mg) which was recrystallized from hot toluene. The infrared absorption spectrum of the isolated compound was identical with that given by 3-methylcatechol. Both the reaction product and authentic 3-methylcatechol melted at 65–66°. A mixture melting point showed no depression.

Experiments with Cell Extract. Crude cell extract would not oxidize benzene unless ethanol was added to the reaction mixture. Such extracts contained an active ethanol dehydrogenase which presumably produced NADH, which is required in the initial reaction. In the absence of ethanol the addition of NAD^+, NADH, ferrous ions and cysteine, either separately or in combination, did not result in benzene oxidation. In the presence of ethanol these cofactors caused only a slight increase in the rate of the reaction. When benzene was incubated anaerobically with crude cell extract no reaction products were detected in the reaction mixture. The extracts were stable for at least 2 months when stored at −15°. On standing at 4° for 24 hr, 55% of the original activity was lost. Heating at 55° for 3 min caused complete inactivation of the enzyme. Some of the substrates oxidized by the crude cell extract are shown in Table III.

Ammonium Sulfate Treatment of Cell Extract. Crude cell extract was treated with protamine sulfate as described in Materials and Methods and brought to 70% saturation with respect to ammonium sulfate. The protein precipitate was dissolved in 0.02 M potassium phosphate buffer (pH 7.2) and desalted by passage through a Sephadex G-25 column. The resulting cell extract was completely inactive when incubated with benzene. The

TABLE V: Cofactor Requirement for Benzene Oxidation.[a]

Additions (3.3×10^{-4} M)	Benzene Oxygenase Act. (μl of O_2/10 min per mg of protein)
None	0.00
Fe^{2+}	0.90
NAD	2.90
NADH	3.00
NADP	0.00
NADPH	0.40
NAD $FeSO_4$	6.40
NADPH $FeSO_4$	1.30

[a] Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 140 μmoles; and cell extract, 11 mg of protein. Benzene, 5.5 μmoles in 0.2 ml of 25% ethanol, was added from the side arm. The enzyme preparation used was the 30–70% ammonium sulfate fraction which was eluted from a Sephadex G-25 column with 0.02 M KH_2PO_4 buffer (pH 7.2) containing 0.01 M L-cysteine. Additions were at the concentration indicated in the table. $NADP^+$, 1.0 μmole; glucose 6-phosphate, 50 μmoles; and glucose 6-phosphate dehydrogenase, 100 units.

addition of NAD^+, 3×10^{-4} M; $NADP^+$, 3×10^{-4} M; FMN, 6×10^{-5} M; FAD, 6×10^{-5} M; tetrahydrofolate, 3×10^{-4} M; or boiled cell extract did not stimulate activity. When the ammonium sulfate extract was incubated with NAD^+ and $FeSO_4$, both at concentrations of 3×10^{-4} M, 14% of the activity originally present in the crude extract was recovered. No further stimulation of actvity was observed upon the addition of any of the above cofactors. Sulfydryl compounds, however, did stimulate activity in the presence of NAD^+ and ferrous ions. Table IV shows the effect of sulfydryl compounds on benzene oxidation. Since the presence of cysteine resulted in the recovery of 84% of the activity originally present in the crude extract, it was routinely added to the cell extract prior to ammonium sulfate fractionation. After dialysis of the ammonium sulfate precipitate for 24 hr against 0.02 M KH_2PO_4 buffer (pH 7.2) containing 0.01 M cysteine, all of the activity in the crude extract was recovered. The effect of NAD^+, $NADP^+$, and their respective reduced counterparts is shown in Table V. NADPH, formed by NADPH-generating system, in the presence of ferrous ions, was approximately one-fifth as active as NADH or NAD^+. The extract used in these experiments was the most active preparation obtained and showed considerable activity in the presence of either NADH or NAD^+ without further additions. With less active cell extracts, ferrous ions usually exhibited a four- to fivefold stimulation of activity (Table VI). Ferric ions and calcium ions also showed stimulatory activity.

TABLE VI: Metal Ion Requirement for Benzene Oxygenase.[a]

Metal Ion Present	μl of O_2 Consumed/10 min per mg of Protein
None	0.47
Fe^{2+} ($FeSO_4$)	2.60
Fe^{3+} ($FeCl_3$)	1.40
Ca^{2+} ($CaCl_2$)	1.13
Co^{2+} ($CoCl_2$)	0.20
Cu^{2+} ($CuCl_2$)	0.38
Mg^{2+} ($MgSO_4$)	0.00
Zn^{2+} ($ZnSO_4$)	0.00

[a] Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 140 μmoles; NAD^+, 1.0 μmoles; and enzyme 30–70% $(NH_4)_2SO_4$ fraction, 11 mg of protein. Benzene, 5.5 μmoles in 0.2 ml of 25% (v/v) ethanol, was added from the side arm. The metal ions were present in a final concentration of 3.3×10^{-4} M.

The optimal pH was found to be 7.5 when examined in Tris buffer (0.1 M). At pH 7.0, 81% and at pH 8.0, 91% of the activity at pH 7.5 were observed.

Partial Purification of Benzene Oxygenase. Crude cell extract, in 0.02 M KH_2PO_4 buffer (pH 7.2) containing 0.01 M cysteine, was treated with protamine sulfate (as described in Materials and Methods), and the resulting extract was brought to 30% saturation with solid ammonium sulfate. The turbid solution was centrifuged at 20,000*g* for 10 min and the precipitate was discarded. The supernatant solution was further fractionated by precipitating at 50 and 70% ammonium sulfate saturation. Each precipitate was dissolved in 0.02 M KH_2PO_4 buffer (pH 7.2) containing 0.01 M cysteine and dialyzed against 20 l. of the same buffer for 18 hr. The activity of each fraction was determined. Oxygen consumption with the 30–50% ammonium sulfate fraction was observed after a lag period of approximately 10 min, while the 50–70% ammonium sulfate fraction was completely inactive. When the two ammonium sulfate fractions were combined, the lag period observed with the 30–50% ammonium sulfate fraction was abolished (Figure 3). Each fraction lost activity when heated at 55° for a period of 3 min. Each fraction was stable at −15° for at least 2 weeks. Attempts to purify the 30–50% ammonium sulfate fraction by assaying in the presence of 50–70% ammonium sulfate fraction resulted in complete loss of activity. Column chromatography with DEAE-cellulose, TEAE-cellulose, CM-cellulose, hydroxylapatite, or Sephadex G-200 failed to yield a preparation which was active in the presence of the 50–70% ammonium sulfate fraction. The 50–70% ammonium sulfate fraction readily lost activity when subjected to chroma-

TABLE VII: Purification of Benzene Oxygenase.[a]

Fraction	Vol (ml)	Protein (mg)	Total Act. (units)[b]	Sp Act. (units/mg ($\times 10^2$)
Crude cell extract	315	5040	87	1.7
Protamine sulfate treatment	350	4708	161	3.4
30–50% $(NH_4)_2SO_4$ precipitate	150	2250	0	0.0
50–70% $(NH_4)_2SO_4$ precipitate	100	1800	0	0.0
Combined $(NH_4)_2SO_4$ precipitate	250	4050	125	3.0
Hydroxylapatite column, 50–70% $(NH_4)_2SO_4$ fraction	90	249	54	21.6

[a] The assay system contained in μmoles/3.0 ml: KH_2PO_4 (pH 7.2), 160; NAD^+, 1.0; $FeSO_4$, 1.0; benzene, 2.25 (in 0.1 ml of 25% ethanol); and cell extract. [b] One unit catalyzes the uptake of 1.0 μmole of oxygen/min based on the initial rate observed in the first 5 min of reaction.

tography with DEAE-cellulose or TEAE-cellulose. A sevenfold purification over the activity observed when the two $(NH_4)_2SO_4$ fractions were combined was achieved by chromatography on hydroxylapatite. In this experiment, the 50–70% ammonium sulfate precipitate (1800 mg in 100 ml of 0.001 M KH_2PO_4 buffer (pH 7.0) containing 0.005 cysteine) was applied to the top of a hydroxylapatite column (15 × 2.5 cm) and eluted at a rate of 41 ml/hr. The column was washed with two column volumes of 0.001 M KH_2PO_4 buffer (pH 7.0) containing 0.005 M cysteine, and 10-ml fractions were collected. A gradient of 0.005–0.05 M KH_2PO_4 buffer (pH 7.0) containing 0.005 M cysteine was used to elute the enzyme. The activity in each tube was assayed manometrically in the presence of freshly thawed 0–50% ammonium sulfate fraction. The activity of the latter fraction did not change over the period of the purification. Active fractions from the hydroxylxapatite column, tubes 27–36 (0.010–0.017 M KH_2PO_4), were pooled. All attempts to purify this fraction resulted in complete loss of activity. A summary of the purification procedure is shown in Table VII.

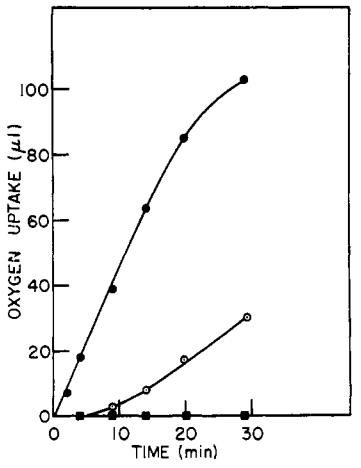

FIGURE 3: Oxidation of benzene by ammonium sulfate fractionated cell extracts. Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 160 μmoles; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; benzene, 2.25 μmoles in 0.1 ml of 25% (v/v) ethanol, and cell extracts as follows: 30–50% $(NH_4)_2SO_4$ fraction, 11 mg of protein; 50–80% $(NH_4)_2SO_4$ fraction, 9.0 mg of protein; 30–50% plus 50–80%, 10 mg of protein. Results are corrected for endogenous respiration in the absence of substrate. (●—●—●) 30–50% plus 50–80% $(NH_4)_2SO_4$; (⊙—⊙—⊙) 30–50% $(NH_4)_2SO_4$; (■—■—■) 50–70% $(NH_4)_2SO_4$.

Active fractions were colored deep red. Since the absorption spectrum indicated the presence of a *c*-type cytochrome, the effect of heme-reactive agents and other inhibitors was investigated. Table VIII shows that cyanide and fluoride did not appreciably inhibit the reaction. The presence of catalase did not affect the rate of oxygen uptake although this enzyme is a potent inhibitor of the hemoprotein tryptophan pyrrolase (Feigelson and Greengard, 1961). Hemin, which is a prosthetic group of bacterial tryptophan pyrrolase, did not stimulate activity of the hydroxylapatite fractions; neither did it restore activity to extracts which were inactive after treatment with DEAE-cellulose. All attempts to purify the fractions obtained after hydroxylapatite chromatography resulted in complete loss of activity. No colored non-heme iron protein, such as rubredoxin (Peterson *et al.*, 1966) and adrenodoxin (Kimura and Susuki, 1967), or putidaredoxin (Cushman *et al.*, 1967), was detected after DEAE-cellulose chromatography. A sample of putsdaredoxin failed to reactivate a DEAE-cellulose-treated extract.

Oxidation of Suspected Intermediates in Benzene Degradation. When 30–70% ammonium sulfate treated extracts (containing 0.01 M cysteine) were incubated with benzene, *cis*-benzene glycol, and catechol, 2.0, 1.5, and 1.0 moles of oxygen per mole of substrate were consumed, respectively (Figure 4). Phenol and *trans*-benzene glycol were metabolized at a much slower rate. The flasks containing benzene, catechol, and *cis*-benzene glycol were all colored deep yellow, and spectroscopic examination showed that the accumulated product from all three substrates had λ_{max} 375 mμ at pH 12.0, which was removed to λ_{max} 317 mμ upon acidification. These

TABLE VIII: Effect of Inhibitors on Benzene Oxygenase Activity.[a]

Inhibitor	Concn (mM)	Act. (% control)
None		100
NaN_3	2.00	62
KCN	2.00	85
KF	2.00	85
$K_3Fe(CN)_6$	0.03	65
FMN	0.03	29
FMN	1.00	0
Methylene blue	3.00	0
2,2′-Bipyridine	2.00	6
o-Phenanthrolene	2.00	29
8-Hydroxyquinoline	2.00	3
EDTA	2.00	3
Tiron	2.00	6
Aminopterin	2.00	97

[a] Warburg flasks contained in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 160 μmoles; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; benzene, 2.25 μmoles (in 0.1 ml of 25% ethanol); and 30–70% $(NH_4)_2SO_4$ fractionated cell extract, 16 mg of protein. Inhibitors were incubated with the cell extract, at the concentrations indicated, for 20 min prior to the addition of NAD^+, $FeSO_4$, and substrate.

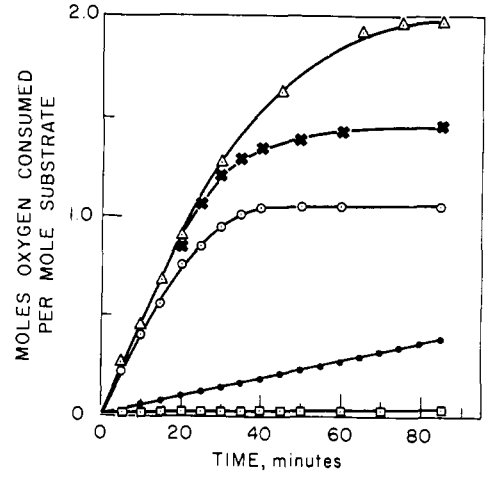

FIGURE 4: Oxidation of suspected intermediates in benzene degradation by 30–70% ammonium sulfate fractionated extract. Warburg flasks contained, in a final volume of 3.0 ml: KH_2PO_4 (pH 7.2), 160 μmoles; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; extract, 15 mg of protein; and benzene, 5.5 μmoles in 0.2 ml of 25% (v/v) ethanol. Other substrates (5 μmoles) were added in 0.5 ml of distilled water. Results are corrected for endogenous respiration in the absence of substrate. (⊙—⊙—⊙) Catechol, (x—x—x) *cis*-benzene gylcol, (△—△—△) benzene, (⊡—⊡—⊡) phenol, and (●—●—●) *trans*-benzene glycol.

spectral changes are identical with those reported for the product formed from catechol by catechol 2,3-oxygenase (Dagley *et al.*, 1960).

Identification of Catechol as an Intermediate in Benzene Degradation. The identification of 2-hydroxymuconic semialdehyde as a product of benzene oxidation by cell extracts indicates that catechol is an intermediate in benzene degradation. Further evidence was obtained from the following experiments with [^{14}C]benzene. The reaction mixture contained in 1.0 ml: 70% ammonium sulfate extract, 15 mg of protein; NAD^+, 1.0 μmole; $FeSO_4$, 1.0 μmole; catechol, 50 μmoles; and [^{14}C]benzene, 0.26 μmole (187,500 cpm). The reaction was started by the addition of catechol and benzene. Incubation was performed at 30° with constant shaking. After 24 min, the reaction was terminated by the addition of 0.2 ml of 3 N HCl. The protein precipitate was removed by centrifugation and the supernatant solution was neutralized with 0.5 N NaOH. The neutral solution was extracted with 5 ml of ethyl acetate. After drying the organic layer over anhydrous Na_2SO_4, the solvent was removed at room temperature and the residue was dissolved in 0.1 ml of acetone. The acetone solution was chromatographed in a band on a thin-layer chromatogram sheet in solvent A. The developed chromatogram was dried at room temperature and placed in contact with Kodak No-Screen X-Ray film for 3 days. Only one radioactive band was observed on the developed radioautograph and this band had an R_F value identical with authentic catechol which was run under the same conditions. The radioactive area was scraped off the plate and the silica gel was washed with three 2-ml portions of ethyl acetate. The ethyl acetate layer was evaporated to dryness at room temperature and the residue was dissolved in 4 ml of methanol. Aliquots were taken for spectroscopic and radioactivity determinations. A total of 34.2 μmoles of catechol (127,200 cpm) was recovered as calculated from absorption at 275 mμ (ϵ_m 275 mμ catechol = 2300). Identity of the radioactive material was confirmed by radioautography. The radioactive compound cochromatographed exactly with synthetic catechol in solvent systems A, R_F 0.46; B, R_F 0.50; and C, R_F 0.27. Additional evidence was obtained by adding the labeled material to synthetic catechol and recrystallizing to constant specific activity.

Identification of cis-Benzene Glycol as an Intermediate in Benzene Degradation. The reaction mixture contained, in micromoles: KH_2PO_4 buffer (pH 7.2), 70; NAD^+, 1.0; *cis*-benzene glycol, 50; [^{14}C]benzene, 0.26 (187,500 cpm); and ammonium sulfate extract, 12 mg of protein; in a final volume of 5.00 ml. Incubation was performed at 30° with constant shaking. The reaction was terminated after 30 min by heating at 70° for 2 min. The precipitated protein was removed by centrifugation and the precipitate was washed twice with 3.0-ml portions of distilled water. The original supernatant solution and the washings were combined and extracted three times with 5.0-ml portions of ethyl acetate. The ethyl acetate extracts were combined and the solvent was removed under vacuum at room temperature. The residue was dissolved in 2.0 ml of distilled water and applied to the top of a Sephadex G-10 column (8 × 1 cm). Water was used to elute the sample from the column. Fractions of 3.0 ml were collected and examined for absorbance at

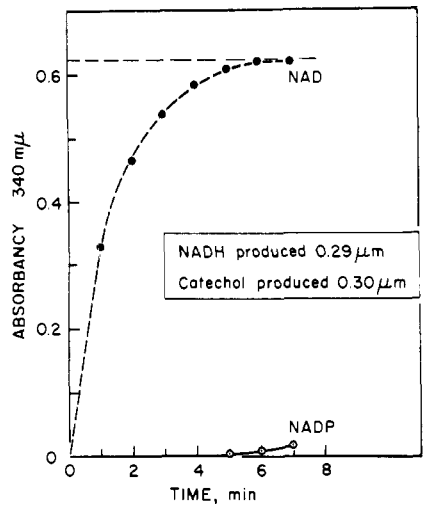

FIGURE 5: Dehydrogenation of *cis*-benzene glycol to catechol by ammonium sulfate treated extract. Anaerobic cuvets contained, in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.2), 250 μmoles; NAD^+, 1.0 μmole; *cis*-benzene glycol, 0.3 μmole; and cell extract, 2.8 mg of protein. A reference cuvet contained all components except for the omission of *cis*-benzene glycol. Cuvets were made anaerobic by alternately evacuating and flushing with argon for 5 min. The reaction was started by the addition of *cis*-benzene glycol from the side arm of the cuvet. The amount of catechol produced in the reaction was measured colorimetrically (16).

260 mμ and for radioactive content. The *cis*-benzene glycol recovered was 4.6 μmoles (16,900 cpm) as judged by absorbance at 260 mμ. Radioautography revealed that the isolated *cis*-benzene glycol and the radioactive material had identical R_F values in solvent systems A, R_F 0.29; B, R_F 0.36; C, R_F 0.15.

The experiment was repeated with *trans*-benzene glycol (50 μmoles) replacing the *cis* isomer as carrier. Incubation and isolation were essentially identical with that described above. In this experiment, the amount of isolated carrier was 47.2 μmoles (100 cpm).

cis-Benzene Glycol Dehydrogenase Activity in Cell Extracts. When *cis*-benzene glycol was incubated with ammonium sulfate treated extract and NAD^+ under anaerobic conditions, it was possible to show NADH formation (Figure 5). In this experiment, *cis*-benzene glycol was stoichiometrically converted into catechol with concomitant formation of equimolar amounts of NADH.

The enzyme catalyzing the dehydrogenation of *cis*-benzene glycol was located in the 50–70% ammonium sulfate fraction. This enzyme proved to be extremely unstable and only a threefold purification was achieved after TEAE-cellulose column chromatography. Addition of FMN (2×10^{-5} M) to the TEAE-treated extract caused a twofold increase in the specific activity. Attempts to purify the enzyme further resulted in a complete loss of activity. Experiments with the preparation obtained from TEAE-cellulose chromatography showed that the rate of the reaction was proportional to enzyme concentration. Michaelis–Menten constants of 6.65×10^{-5} and 5.70×10^{-4} were obtained for *cis*-benzene glycol and NAD^+, respectively. The enzyme preparation was specific for *cis*-benzene glycol, the *trans* isomer being completely inert. Attempts to substitute $NADP^+$ for NAD^+ were unsuccessful.

Discussion

Evidence presented in this paper suggests that benzene, in the presence of NAD^+, ethanol, ferrous ions, and cysteine, is oxidized with the uptake of 1 mole of oxygen/mole of substrate. The first detectable product of the oxygenase reaction is *cis*-benzene glycol, and the NAD^+-dependent dehydrogenation of this compound to catechol has been demonstrated.

The failure of NADH and ferrous ions to promote benzene oxidation is probably due to the presence of an active NADH oxidase in the enzyme preparations. Since the presence of alcohol and NAD^+ results in the formation of NADH and stimulates the oxidation of benzene this indicates that NADH is probably one of the cofactors required for the initial reaction. However, at the present time it is not possible to state conclusively whether NAD^+ or NADH is the actual cofactor in benzene oxidation.

Theories as to the intermediates involved in the formation of catechol from benzene are illustrated in Figure 1. If the initial attack on the benzene nucleus produced an epoxide, this reaction would be described as a mixed-function oxidation (Mason, 1956). Such reactions are characterized by the consumption of 1 mole each of oxygen and reduced pyridine nucleotide per mole of substrate. One atom of molecular oxygen is incorporated into the substrate while the other is reduced to water. Although benzene is oxidized with the consumption of 1 mole of oxygen/mole of substrate and a requirement for an NADH-generating system has been indicated, it seems unlikely that epoxidation is involved in the early stages of benzene oxidation of *P. putida*. Epoxides invariably undergo hydrolytic fission with the formation of *trans*-glycols. Failure of washed cell suspensions and cell extracts to metabolize *trans*-benzene glycol at a significant rate would seem to exclude epoxidation as a reaction in benzene oxidation. It has been demonstrated that 1,2-epoxycyclohexa-3,5-diene (benzene epoxide) is an extremely unstable compound which rapidly isomerizes to phenol under the mildest conditions (Vogel and Gunther, 1967). If benzene epoxide is an intermediate in benzene metabolism, it should be possible to detect phenol in the reaction mixtures. Phenol has never been detected in our system, and failure of cell extracts to metabolize this compound suggests that monohydroxylation of the benzene nucleus is not involved in catechol formation.

Identification of *cis*-benzene glycol as an intermediate in benzene metabolism presents a new aspect of biological hydroxylation. Previous work on the mechanism of hydroxylation of kynurenic acid (Taniuchi and Hayaishi, 1963) has shown that 7,8-dihydro-7,8-dihydroxykynurenic acid is the first identifiable product when kynurenic acid is hydroxylated by cell extracts, prepared from *P. fluorescens* adapted to tryptophan. This compound is presumably the *trans* isomer, since kynurenic acid 7,8-oxide has been proposed as the initial product formed from kynurenic acid. Walker and Wiltshire

(1953) isolated D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from cultures of *Bacillus naphthalinicum nonliquefans* growing at the expense of naphthalene. This compound was identified as a product of naphthalene oxidation by cell extracts prepared from a soil pseudomonad grown with naphthalene (Griffiths and Evans, 1965). Studies with $^{18}O_2$ have shown that the enzymatic fixation of oxygen into the naphthalene nucleus is almost certainly a mixed-function oxidation (Holtzman *et al.*, 1967). The authors demonstrated the incorporation of only one atom of the isotopic oxygen molecule into 1,2-dihydro-1,2-dihydroxynaphthalene. This result, obtained with an enzyme preparation prepared from mouse liver microsomes, suggests that naphthalene undergoes oxygen fixation to form 1,2-dihydro-1,2-epoxynaphthalene. The latter compound then undergoes hydrolytic cleavage of the epoxide to form 1,2-dihydro-1,2-dihydroxynaphthalene. Epoxide formation followed by hydrolysis to a "diol" has been suggested as a mechanism for catechol formation from benzoic acid (Taniuchi *et al.*, 1964). These results are all at variance with our observations. The formation of *cis*-benzene glycol may involve the prior formation of a peroxide (Figure 6). Such a reaction has been proposed for the enzymatic formation of catechol from anthranilic acid (Kobayashi *et al.*, 1964). Further proof awaits purification of the benzene oxygenase system and studies with $^{18}O_2$.

The presence of *cis*-benzene glycol dehydrogenase activity in cell extracts of *P. putida* contrasts with observations in mammalian systems. Sato *et al.* (1963) detected *trans*-benzene glycol in the urine of rabbits dosed with benzene and showed that this compound was further metabolized to catechol. An enzyme catalyzing the NADP-dependent dehydrogenation of *trans*-benzene glycol to catechol has been partially purified from rabbit liver (Ayengar *et al.*, 1959). The enzyme reported in this paper is specific for NAD^+ and *cis*-benzene glycol.

The enzyme(s) catalyzing the initial oxidation of benzene appears to be similar to the enzyme(s) responsible for the hydroxylation of kynurenic acid (Taniuchi and Hayaishi, 1963). Both require NADH, or an NADH-generating system, ferrous ions, and L-cysteine for maximal activity. However, the kynurenic acid hydroxylase has been shown to consist of a single protein with bound FMN as a cofactor (Mori *et al.*, 1966). Similarly, purified salicylate hydroxylase (Yamamoto *et al.*, 1965) and *p*-hydroxybenzoate hydroxylase (Hosokawa and Stanier, 1966) have been shown to contain 1 mole of FAD/mole of enzyme protein. Hydroxylating systems containing more than one enzyme have been demonstrated for octane hydroxylation by *Pseudomonas oleovorans* (Peterson *et al.*, 1966), 2-bornanone 5-exohydroxylation by *P. putida* (Cushman *et al.*, 1967), and adrenal steroid 11β hydroxylation by adrenal mitochondria (Kimura, 1966). These systems appear to contain a pyridine nucleotide dehydrogenase which transfers electrons to specific non-heme ferroproteins, which in turn reduce the hydroxylating enzymes. The enzyme system responsible for the oxygenation of benzene may be similar to these mechanisms. However, the failure of putidaredoxin to stimulate DEAE-treated cell extracts

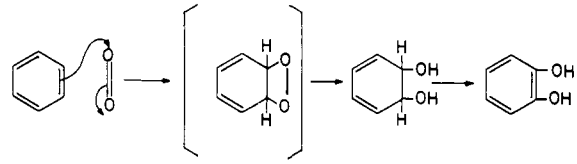

FIGURE 6: Proposed pathway for catechol formation from benzene.

would suggest that the double hydroxylation of benzene is different in systems involving monohydroxylation. Further proof awaits the development of a sensitive assay and purification of the enzyme system. Such studies are in progress.

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Spin-Labeled Hemoglobin Subunits*

Shun-ichi Ohnishi, Toyozo Maeda, Tadanao Ito, Kun-Joo Hwang, and Itiro Tyuma

ABSTRACT: The isolated subunits from human adult hemoglobin and their mercurated derivatives in carbon monoxy form have been spin labeled with *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide. The electron spin resonance spectra of the labeled subunits and their various mixtures have been measured at room temperatures. The sedimentation coefficients, the number of the bound labels, and the number of the reactive SH groups have also been determined. The spectra of all the labeled subunits are similar and are very different from that of the spin-labeled tetramer $\alpha_2\beta_2$. The spin label attached to the β93 SH group in the isolated β chains gives a different spectrum from that of the same label attached to the same SH group in the tetramer, indicating that the conformation of the β chains in the isolated form differs from that in the $\alpha_2\beta_2$ tetramer. When the labeled β chains are mixed with native α chains, the spectrum of the former changes very rapidly (within 1 sec) into the spectrum indistinguishable from that of the tetramer. The normally functioning conformation of the β chains is achieved only when they combine with the α chains of different composition.

The idea that the functional properties of hemoglobin are based upon the conformation or conformational changes in the constituent subunits seems to be receiving more and more supporting evidence from various studies (Muirhead and Perutz, 1963; Perutz and Mazzarella, 1963; Perutz *et al.*, 1964). Better understanding of these properties can be achieved by investigating the structures of the isolated subunits and the interactions between these subunits. Bucci and Fronticelli (1965) succeeded in isolating the α and β chains of human hemoglobin after treatment with PMB[1] and the Rome group studied several properties of each subunit and of reconstituted hemoglobins (Bucci *et al.*, 1965; Antonini *et al.*, 1965, 1966). Their method of mercury removal was not satisfactory, however, and Tyuma *et al.* (1966) have presented an improved method for the complete removal using SH Sephadex and mercaptoethanol. The latter authors have also investigated some physicochemical and physiological properties of the subunits thus obtained (Tyuma *et al.*, 1966; Beychok *et al.*, 1967).

The spin-label technique has been successfully applied to the study of conformational changes in hemoglobins (Boeyens and McConnell, 1966; Ohnishi *et al.*, 1966; Ogawa and McConnell, 1967). This technique is unique, compared with other usual physicochemical methods, in that the label reports its *local* environment or changes in the environment through the correlation time of molecular tumbling. In the present investigation, we used this technique to elucidate conformational properties of the subunits of human hemoglobin and of the reconstituted hemoglobin.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMB, *p*-mercuribenzoate; α_{PMB} and β_{PMB}, mercurated α and β chains; α and β, demercurated α and β chains; $\alpha_2\beta_2$ or Hb A, parent human adult hemoglobin tetramer; the superscript * indicates that the protein has been spin labeled.