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Purification and Propeties of (+)-*cis*-Naphthalene Dihydrodiol Dehydrogenase of *Pseudomonas putida*

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Cells of *Pseudomonas putida*, after growth with naphthalene as sole source of carbon and energy, contain an enzyme that oxidizes (+)-*cis*-1(R),2(S)-dihydroxy-1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene. The purified enzyme has a molecular weight of 102,000 and apparently consists of four 25,500 molecular weight subunits. The enzyme is specific for nicotinamide adenine dinucleotide as an electron acceptor and also oxidizes several other *cis*-dihydrodiols. However, no enzymatic activity was observed with *trans*-1,2-dihydronaphthalene, or the K-region *cis*-dihydrodiols of carcinogenic polycyclic hydrocarbons.

Mammals and microorganisms oxidize naphthalene to 1,2-dihydroxynaphthalene. Liver microsomes incorporate one atom of molecular oxygen into naphthalene to yield 1,2-naphthalene oxide (21). The latter compound can undergo several metabolic reactions, one of which is the enzymatic addition of water by the enzyme epoxide hydrase to form *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (*trans*-naphthalene dihydrodiol) (30). Ayengar et al. (3) partially purified an oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent enzyme from rabbit liver that oxidized *trans*-3,5-cyclohexadiene-1,2-diol (*trans*-benzene dihydrodiol) to 1,2-dihydroxybenzene (pyrocatechol). The same enzyme preparation also oxidized naphthalene dihydrodiol (presumably the *trans*-isomer), although the reaction product was not characterized. Jerina et al. (22) showed that liver microsomes from rat, rabbit, guinea pig, and mouse converted naphthalene and 1,2-naphthalene oxide to *trans*-naphthalene dihydrodiol in which there was an excess of the (−) enantiomer. Interestingly, the $NADP^+$-dependent oxidoreductase from rabbit liver oxidized the (+) enantiomer at a rate severalfold greater than the (−) enantiomer. The significance of this observation remains to be established.

Bacteria oxidize naphthalene by a different reaction sequence than that described above for liver microsomes. Murphy and Stone (27) showed that 1,2-naphthoquinone accumulated in culture filtrates when a *Pseudomonas* species was grown with naphthalene as sole source of carbon and energy. Subsequent studies by Evans and colleagues (7, 10) demonstrated that cells of *Pseudomonas* sp. NCIB 9816, after growth on naphthalene, contained an enzyme that oxidized 1,2-dihydroxynaphthalene. The first detectable product in the oxidation of naphthalene by *Pseudomonas putida* has been identified as (+)-*cis*-1(R),2(S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (20). Both atoms of oxygen in this product are derived from a single molecule of oxygen (unpublished results). The formation of *cis*-naphthalene dihydrodiol during the oxidation of naphthalene by *Pseudomonas* sp. NCIB 9816 has also been reported (4). These observations cast doubt on earlier reports that bacteria oxidize naphthalene in a manner analogous to that demonstrated in mammalian microsomes (36, 38). *cis*-Dihydrodiols have been identified as intermediates in the bacterial oxidation of benzene (12, 15, 19), toluene (14, 24, 40), *p*-chlorotoluene (16), ethylbenzene (13), biphenyl (17), benzoic acid (33), and 5-amino-4-chloro-2-phenyl-3-(2H)-pyridazinone (9). In almost every case an oxidized nicotinamide adenine dinucleotide-specific oxidoreductase was demonstrated to catalyze the oxidation of the *cis*-dihydrodiols to the corresponding catechol derivatives. The enzymes catalyzing the oxidation of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (benzoid acid dihydrodiol) (32) and *cis*-benzene dihydrodiol (2) have been purified and their properties have been described.

We now wish to report the purification and properties of (+)-*cis*-naphthalene dihydrodiol dehydrogenase. This enzyme is induced in cells of *Pseudomonas putida* when the organism is

grown with naphthalene as the sole source of carbon and energy.

MATERIALS AND METHODS

Chemicals. (+)-*cis*-Naphthalene dihydrodiol was isolated from culture filtrates after *P. putida* strain NP-119 was grown on glucose in the presence of naphthalene. In a typical experiment the organism was grown in 10 liters of mineral salts medium (35) supplemented with 0.2% glucose and 0.2% naphthalene. Growth was carried out in a New Brunswick model M-14 microferm fermentor. After 17 h the cells were removed by centrifugation and the clear supernatant solution was extracted twice with 4 liters of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate, and the solvent was removed to leave 2.7 g of an oil. The oil was recrystallized from *n*-hexane to give 600 mg of pure (+)-*cis*-naphthalene dihydrodiol. The physical properties of the isolated material were identical to those reported previously (20). *cis*-3-Phenyl-3,5-cyclohexadiene-1,2-diol(*cis*-biphenyl dihydrodiol) was prepared as described previously (17). *Beijerinckia* strain B8-36 was used to prepare *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (*cis*-phenanthrene dihydrodiol) and *cis*-1,2-dihydroxy-1,2-dihydroanthracene (*cis*-anthracene dihydrodiol). The procedure used was analogous to that described for the isolation of *cis*-biphenyl dihydrodiol. *cis*-3,5-Cyclohexadiene-1,2-diol (*cis*-benzene dihydrodiol), *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (*cis*-toluene dihydrodiol), and *cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol (*cis*-ethylbenzene dihydrodiol) were prepared as previously described (12–14). *P. putida* strain 39/D, which was used to produce the above monocyclic *cis*-dihydrodiols, was also used to prepare *cis*-3-methyl-6-chloro-3,5-cyclohexadiene-1,2-diol (*cis*-*p*-chlorotoluene dihydrodiol), *cis*-3-methyl-6-bromo-3,5-cyclohexadiene-1,2-diol (*cis*-*p*-bromotoluene dihydrodiol), and *cis*-3-methyl-6-fluoro-3,5-cyclohexadiene-1,2-diol (*cis*-*p*-fluorotoluene dihydrodiol). *cis*-Benz(a)anthracene-5,6-dihydrodiol, *cis*-benz(a)pyrene-4,5-dihydrodiol, *cis*-7,12-dimethylbenz(a)anthracene-4,5-dihydrodiol, *cis*-3-methylcholanthrene-11,12-dihydrodiol, and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene dihydrodiol were generous gifts from D. M. Jerina, National Institutes of Health. 1,2-Dihydroxynaphthalene was prepared by the method of Corner and Young (5) and purified by vacuum sublimation. NAD^+, $NADP^+$, diethylaminoethyl (DEAE)-cellulose, and DEAE-Sephadex A50 were from Sigma Chemical Co. Sephadex G200 was obtained from Pharmacia. All other materials were of the highest purity commercially available and were used without further purification.

Organisms. *P. putida* strain NP was isolated from soil by enrichment culture on naphthalene as sole source of carbon and energy. *P. putida* strain NP-119 is a mutant strain that oxidizes naphthalene to *cis*-naphthalene dihydrodiol (20). *P. putida* strain 39/D is a mutant that oxidizes monocyclic aromatic hydrocarbons and their halogenated derivatives to *cis*-dihydrodiols (11). *Beijerinckia* strain B-836 is a mutant that oxidizes biphenyl (17), phenanthrene, and anthracene (unpublished observations) to *cis*-dihydrodiols.

Growth of organisms. *P. putida* strain NP was grown in a 750-liter fermentor by Truett Laboratories, Biochemical Division, Dallas, Tex. The growth medium contained per liter: naphthalene, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; and $FeSO_4 \cdot 7H_2O$, 12 mg. The pH of the medium was adjusted to 7.2 with 5 N NaOH prior to sterilization and the addition of $MgSO_4$ and $FeSO_4$. Cells were harvested at the end of the log phase of growth and stored at −15 C until required. The other organisms were grown as described previously (14, 17).

Preparation of cell extract. Washed cells of *P. putida* strain NP were suspended in 0.02 M KH_2PO_4 buffer, pH 7.2, containing 0.001 M 2-mercaptoethanol (1.0 g wet weight per 3.0 ml of buffer), and disrupted by sonic oscillation in a Biosonik III ultrasonic disintegrator. Cell debris was removed by centrifugation at 13,000 × *g* for 30 min. The clear supernatant solution was centrifuged at 105,000 × *g* for 60 min. The supernatant solution obtained was the crude cell extract.

Protein in cell extracts was determined by the biuret procedure (18) and also by the method of Lowry et al. (26). Bovine serum albumin was used as a standard.

Assay for cis-naphthalene dihydrodiol dehydrogenase. Enzyme activity was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm. Reaction mixtures (3.0 ml) contained in micromoles: KH_2PO_4 buffer (pH 7.0), 270; NAD^+, 8.0; *cis*-naphthalene dihydrodiol, 1.0; and enzyme (0.16 to 0.80 units). The reaction was initiated by the addition of substrate (*cis*-naphthalene dihydrodiol). Under these conditions the rate of the reaction was proportional to enzyme concentration. Initial velocity measurements were made under aerobic conditions and were obtained by adjusting the enzyme concentration so that the recorded curves gave slopes of 40 to 60°. Tangents drawn to the recorded curves obtained during the first 15 s of the reaction were used to calculate the initial velocity. Reactions were performed in duplicate. Certain experiments (see Results) were run under anaerobic conditions; in this case cuvettes were alternately evacuated and flushed with nitrogen for 15 min prior to the addition of substrate.

One unit of enzyme activity was defined as the amount of enzyme required to reduce 1.0 μmol of NAD^+ per min. Specific activities are expressed as units per milligram of protein.

Purification of cis-naphthalene dihydrodiol dehydrogenase. All procedures were performed at 0 to 5 C. Phosphate buffer refers to 0.02 M KH_2PO_4 buffer (pH 7.2) containing 0.001 M 2-mercaptoethanol.

Step 1. To the crude cell extract was added 0.1 volume of a 2% solution of protamine sulfate. The resulting precipitate was removed after centrifugation at 37,000 × *g* for 30 min.

Step 2. The clear supernatant solution obtained from step 1 was brought to 50% saturation by the

careful addition of powdered ammonium sulfate. The precipitated protein was removed after centrifugation at 37,000 × g for 30 min. Ammonium sulfate was added to the clear supernatant solution until 75% saturation was achieved. A repeat centrifugation gave a precipitate which was dissolved in a small amount of phosphate buffer. The concentrated protein solution was dialyzed for 8 h against 10 liters of phosphate buffer.

Step 3. The dialyzed protein solution was applied to the top of a DEAE-cellulose column (4 by 50 cm). The column was washed with phosphate buffer, and 3.5-ml fractions were collected. Fractions 135 to 200 were pooled and dialyzed for 8 h against 10 liters of 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0), containing 0.001 M 2-mercaptoethanol (Tris buffer).

Step 4. The dialyzed solution from step 3 was applied to a DEAE-Sephadex A50 column (1.5 by 7.0 cm). The column was washed with Tris buffer until no protein was detected in the eluate; 3.0-ml fractions were collected. This procedure was repeated with 0.15 M Tris buffer. At this time a linear gradient of 0.15 to 0.20 M Tris buffer was applied to the column. Those fractions (168 to 196) that gave a constant specific activity were pooled and used as a source of pure enzyme.

Polyacrylamide gel electrophoresis. The purified enzyme was examined by disc-gel electrophoresis by the method of Davis (8). Electrophoresis was performed with 7% gels and Tris-glycine buffer (pH 9.5), at a current of 4 mA/gel. Experiments were run at room temperature and terminated when the bromophenol blue marker reached the lower end of the gel (approximately 2 h). Protein was stained by immersing the gel in a 1% solution of aniline blue black in 7% acetic acid. Excess stain was removed electrophoretically.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% gels by the procedures of Weber and Osborn (39).

Ultracentrifugation. The sedimentation velocity of the purified enzyme was determined from the Schlieren patterns obtained when a solution (4.5 mg of protein per ml) of the enzyme was centrifuged at 20 C and 59,780 rpm in a Spinco model E ultracentrifuge.

Molecular weight. The molecular weight of *cis*-naphthalene dihydrodiol dehydrogenase was determined by Sephadex gel filtration (G200) as described by Andrews (1).

Antisera. Antisera to the purified *cis*-naphthalene dihydrodiol dehydrogenase and the crude cell extract were prepared in random-bred New Zealand rabbits. Five 100-mg injections of the purified enzyme in Freund adjuvant (Difco) were given subcutaneously at 2-week intervals. Beginning 1 week after the final injection, the animals were bled at weekly intervals by marginal ear vein puncture. Antisera to the crude cell extract was prepared in a similar manner. A total of 4.0 mg of crude cell protein was used as the antigen. At the time of bleeding, the animals were given booster injections of 6.0 mg of protein.

The gamma globulin (IgG) fraction from the rabbit sera was obtained by precipitation with sodium sulfate (23) followed by chromatography on DEAE-cellulose (25).

Immunoelectrophoresis. The purified enzyme was subjected to immunoelectrophoresis in 60 mM Tris-barbital buffer (pH 8.6) by the method of Scheidegger (34). Precipitin bands were developed with the IgG fraction of antiserum to the purified enzyme and also antiserum to the crude cell extract.

RESULTS

Enzyme purification. A summary of the purification procedure is shown in Table 1. At pH 7.2 the enzyme did not bind to DEAE-cellulose, and elution with 0.02 M KH_2PO_4 buffer resulted in a 45-fold increase in specific activity with a 67% recovery of enzyme units. Further chromatography on DEAE-Sephadex A50 at pH 8.0 gave a homogeneous enzyme preparation. The enzyme was eluted from the column with 0.165 to 0.180 M Tris-hydrochloride buffer (pH 8.0). Fractions that gave a constant specific activity were pooled and used as a source of pure enzyme. The elution profile of protein and naphthalene dihydrodiol dehydrogenase activity is shown in Fig. 1. A 134-fold purification of the enzyme was achieved, with a final yield of 26%.

The purity of the enzyme preparation was deduced from the following criteria. (i) Polyacrylamide gel electrophoresis at pH 9.5 revealed a single band that stained for protein (Fig. 2). (ii) Immunoelectrophoresis of the purified enzyme gave a single precipitin arc with antiserum to the crude cell extract. (iii) Antiserum to the purified enzyme gave a single precipitin arc on immunoelectrophoresis with the purified protein (the low level of enzyme protein in the crude cell extract precluded the observation of a precipitin arc with antisera to the purified enzyme). (iv) The Schlieren profile observed when the purified enzyme was subjected to ultracentrifugal analysis showed a single symmetrical peak with a sedimentation coefficient ($s_{20,w}$) of 6.22. These observations indicate that the *cis*-naphthalene dihydrodiol dehydrogenase obtained from the DEAE-Sephadex column was a homogeneous protein.

Molecular weight and subunit structure. The molecular weight of purified *cis*-naphthalene dihydrodiol dehydrogenase was determined by gel filtration on a standardized column of Sephadex G200. From the results obtained (Fig. 3), the molecular weight of the purified enzyme was estimated to be 102,000.

The subunit composition of the purified enzyme was determined by disc-gel electrophoresis analysis of the reduced enzyme in the pres-

TABLE 1. *Purification of cis-naphthalene dihydrodiol dehydrogenase*

Step	Vol (ml)	Protein (mg)	Units[a]	Sp act[b]	Purification	Recovery (%)
Crude cell extract	1,340	23,450	1,488	0.063		100
1. Protamine sulfate	1,400	18,000	1,456	0.080	1.3	97
2. Ammonium sulfate (50–75%)	99	4,554	1,224	0.270	4.3	83
3. DEAE-cellulose	246	349.3	998	2.850	45.3	67
4. DEAE-Sephadex A50	92.5	45.6	384	8.430	133.8	25.8

[a] One unit is the amount of enzyme required to reduce 1 μmol of NAD^+ per min under standard assay conditions.

[b] Units per milligram of protein.

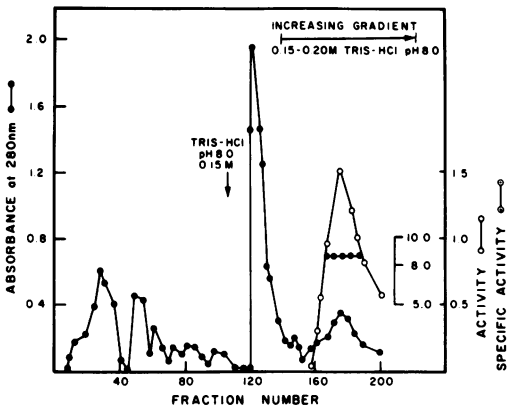

FIG. 1. *Elution profile of cis-naphthalene dihydrodiol dehydrogenase from a column of DEAE-Sephadex A50. Protein was determined by absorbance at 280 nm. Activity and specific activity are defined in the text.*

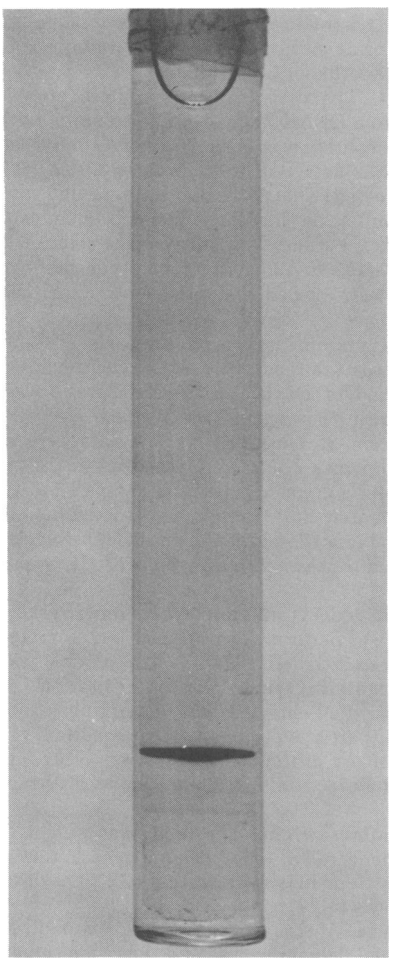

FIG. 2. *Polyacrylamide gel electrophoresis of purified cis-napthalene dihydrodiol dehydrogenase. The enzyme preparation (60 μg of protein) was subjected to electrophoresis for 2 h at 4 mA in Tris-glycine buffer (pH 9.5). The protein migrated from the cathode (top) to the anode.*

ence of 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol. A single protein band was obtained. The molecular weight of the subunits was determined by comparing the migration of the reduced enzyme with the migration of other proteins of known molecular weight. As shown in Fig. 4, the subunit molecular weight was calculated to be 25,500. Thus, the purified naphthalene dihydrodiol dehydrogenase appears to be a tetramer of subunits with identical molecular weight.

Properties of naphthalene dihydrodiol dehydrogenase. The pure enzyme, when stored at 4 C, showed little loss in activity over an 11-day period. After this time, enzymatic activity decreased rapidly. The loss in enzymatic activity could be reduced by storing the enzyme in the presence of 0.001 M 2-mercaptoethanol. Under these conditions, only 20% of the enzyme activity was lost over a 20-day period.

The pH optimum for enzymatic activity was 9.0 (Fig. 5). Consistently higher activity was observed with Tris-hydrochloride and glycine-NaOH buffers than with phosphate buffer. However, the reaction product, 1,2-dihydroxynaphthalene, rapidly autooxidizes to 1,2-naphthoquinone at alkaline pH, and the ab-

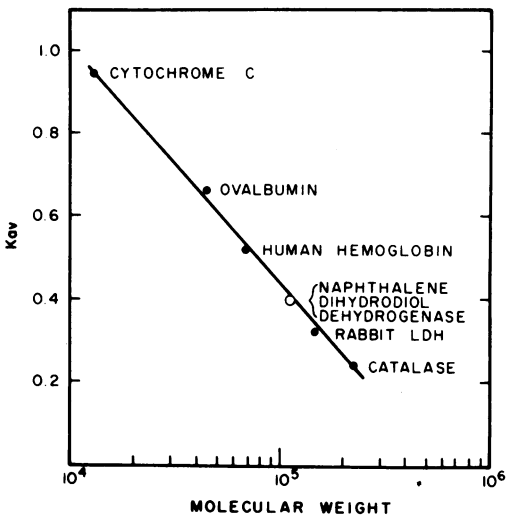

FIG. 3. *Molecular weight of purified cis-naphthalene dihydrodiol dehydrogenase as determined by Sephadex G200 chromatography. The K_{av} values for the proteins of known molecular weight and the purified enzyme were calculated from the formula K_{av} = (Ve − Vo)/(Vt − Vo), as described in the Pharmacia manual, where Ve is the elution volume, Vo is the void volume determined with blue dextran 2,000, and Vt is the bed volume.*

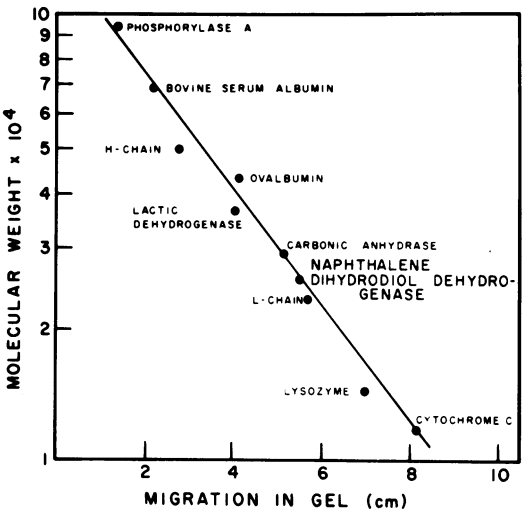

FIG. 4. *Molecular weight of the subunits of purified cis-naphthalene dihydrodiol dehydrogenase as determined by SDS polyacrylamide gel electrophoresis. Separate determinations were made for each protein. Each gel was subjected to electrophoresis at 8 mA/gel for 3.5 h.*

sorption of this product at 340 nm interferes with the enzyme assay. Consequently, the pH studies were performed under anaerobic conditions. In most of the experiments described,

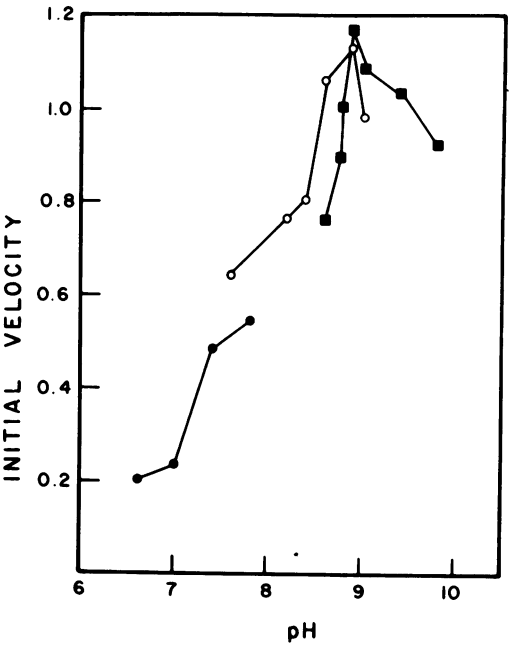

FIG. 5. *pH optimum for purified cis-naphthalene dihydrodiol dehydrogenase. The standard assay system was used with the exception that 0.1 M buffers were used. Each cuvette was made anaerobic by alternately evacuating and flushing with nitrogen for 15 min. Each reaction was initiated by tipping cis-naphthalene dihydrodiol from the side arm of the cuvette. Initial velocity measurements are recorded as changes in absorbance at 340 nm per min. Symbols: ●, KH_2PO_4 buffer; ○, Tris-hydrochloride buffer; ■, glycine-NaOH buffer.*

initial velocity measurements were made under aerobic conditions at pH 7.0. At this pH and during the first minute of the reaction, the autooxidation of 1,2-dihydroxynaphthalene does not interfere with the measurement of enzymatic activity.

At a final concentration of 10^{-3} M, the metal ions Ca^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, and Fe^{3+} did not show any significant effect on enzymatic activity. However, 2,2′-dipyridyl, 8-hydroxyquinoline, and ethylenediaminetetraacetic acid, at a final concentration of 1.67×10^{-2} M, inhibited the enzyme by 55, 25, and 17%, respectively. In these experiments the enzyme was preincubated with the inhibitor for 60 min at room temperature prior to the addition of the substrate. Under the same conditions, *p*-chloromercuribenzoate inhibited the enzyme by 87%. The inhibitions observed are expressed as a percentage of a control experiment in which no inhibitor was present.

The pure enzyme showed an absolute requirement for NAD^+ as the electron acceptor. No

activity was observed when NAD^+ was replaced by $NADP^+$, potassium ferricyanide, methylene blue, or 2,6-dichlorophenolindophenol.

Kinetic analysis revealed that the enzyme has a higher affinity for *cis*-naphthalene dihydrodiol than for NAD^+. The apparent K_m values for NAD^+ and *cis*-naphthalene dihydrodiol were calculated from double reciprocal plots to be 8.0×10^{-4} M and 2.9×10^{-5} M, respectively.

Some of the substrates oxidized by the enzyme are shown in Table 2. The final concentration of each substrate in the reaction mixture was 0.33 mM. All the *cis*-dihydrodiols that served as substrates were produced microbiologically. No enzymatic activity was observed with *trans*-naphthalene dihydrodiol, *cis*-benz(a)anthracene-5,6-dihydrodiol, *cis*-benz(a)pyrene-4,5-dihydrodiol, *cis*-7,12-dimethylbenz(a)anthracene, dihydrodiol, *cis*-3-methylcholanthrene-11,12-dihydrodiol, *cis*-*p*-chlorotoluene-dihydrodiol, *cis*-*p*-bromotoluene dihydrodiol, *cis*-*p*-fluorotoluene dihydrodiol, *cis*-cyclohexane dihydrodiol, *cis*-3-methylcyclohexane diol, ethanol, glycerol, and quinic acid. Data to be published elsewhere show that the enzyme is specific for the (+)-isomer of *cis*-naphthalene dihydrodiol.

Identification of reaction products. When *cis*-naphthalene dihydrodiol and NAD^+ were incubated under aerobic conditions with the pure enzyme, a rapid reduction of NAD^+ was observed. After 1 min the NADH was slowly reoxidized (Fig. 6), and the reaction mixture became yellow. When NADH oxidation ceased, the reaction mixture was extracted with 2 volumes of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate, and the solvent was removed to leave a yellow residue. The residue was dissolved in 50 ml of methanol, and the absorption of this solution was recorded between 230 and 400 nm (Fig. 7). An identical spectrum was given by the autooxidation product formed from synthetic 1,2-dihydroxynaphthalene (Fig. 8) and also by synthetic 1,2-naphthoquinone. The ability of naphthoquinone to non-enzymatically oxidize NADH has been reported (37). When the enzymatic oxidation of *cis*-naphthalene dihydrodiol is performed under anaerobic conditions, autooxidation of 1,2-dihydroxynaphthalene does not occur and it is possible to demonstrate the equimolar production of NADH in the presence of *cis*-naphthalene dihydrodiol (Table 3). Under anaerobic conditions it is also possible to show that equimolar amounts of 1,2-dihydroxynaph-

TABLE 2. *Substrate specificity of cis-naphthalene dihydrodiol dehydrogenase*

SUBSTRATE	ACTIVITY[a]
cis - Naphthalene dihydrodiol	100
cis - Anthracene dihydrodiol	56
cis - Phenanthrene dihydrodiol	53
cis - Biphenyl dihydrodiol	43
cis - Ethylbenzene dihydrodiol	48
cis - Toluene dihydrodiol	26
cis - Benzene dihydrodiol	4

[a]Expressed as a percentage of the activity observed with *cis*-naphthalene dihydrodiol. Initial velocity measurements were obtained as described in Materials and Methods.

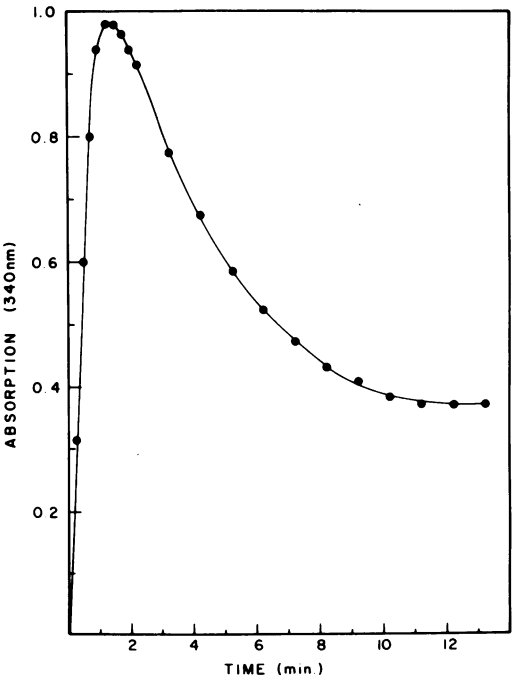

FIG. 6. *Oxidation of cis-naphthalene dihydrodiol by purified enzyme under aerobic conditions. The standard assay system with 72 μg of pure enzyme was utilized. A reference cuvette contained all components except cis-naphthalene dihydrodiol. The reaction was initiated by the addition of substrate, and the absorption changes at 340 nm were recorded by a Cary model 14 recording spectrophotometer.*

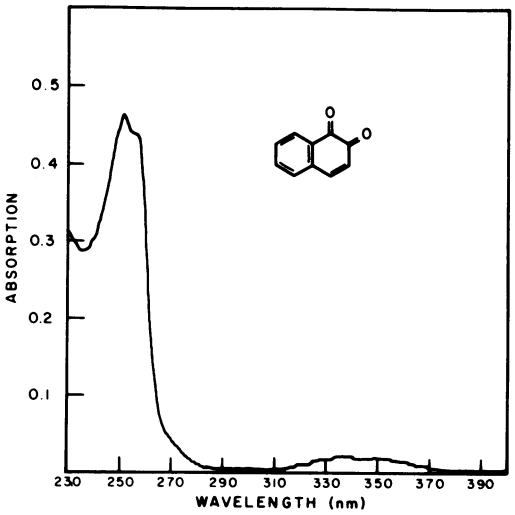

FIG. 7. *Absorption spectrum of the product formed under aerobic condition from cis-naphthalene dihydrodiol. The product obtained from the experiment shown in Fig. 7 was dissolved in 50 ml of methanol, and the spectrum was recorded by a Cary model 14 recording spectrophotometer.*

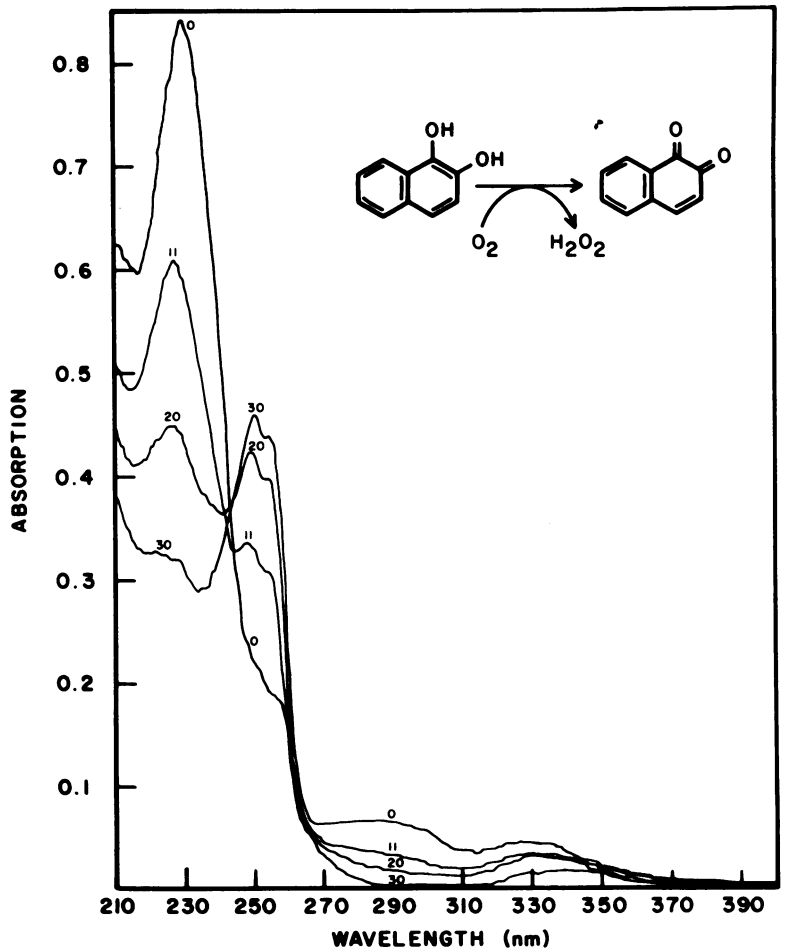

FIG. 8. *Spectral changes observed during the nonenzymatic autooxidation of 1,2-dihydroxynaphthalene. 1,2-Dihydroxynaphthalene (0.06 μmol in 60 μliters of anhydrous tetrahydrofuran) was placed in the side arm of a cuvette. The main compartment contained 3.0 ml of 0.1 M KH_2PO_4 buffer (pH 7.0). The cuvette was alternately evacuated and flushed with nitrogen for 20 min. The 1,2-dihydroxynaphthalene was added to the phosphate buffer, and the absorption spectrum was recorded against a reference cuvette that was treated in an identical manner except for the omission of 1,2-dihydroxynaphthalene. Each cuvette was made aerobic, and spectral changes were recorded at the time intervals shown (minutes).*

TABLE 3. *Quantitative formation of NADH during the enzymatic oxidation of cis-naphthalene dihydrodiol*[a]

cis-Naphthalene dihydrodiol added (μmol)	NAD^+ added (μMOL)	NADH formed (μmol)
0.10	6.00	0.099
0.20	6.00	0.193
0.30	6.00	0.310
0.40	6.00	0.385

[a] Reaction mixtures contained in a final volume of 3.0 ml: KH_2PO_4 buffer (pH 7.0), 270 μmol; purified enzyme (30 μg of protein); and the amounts of *cis*-naphthalene dihydrodiol and NAD^+ as shown. Reactions were run under anaerobic conditions as described in Fig. 9. The amount of NADH formed was calculated using ϵ_{340} = 6,220. The assumption was made that reactions went to completion and a correction was applied to account for the absorption at 340 nm due to the second product of the reaction, 1,2-dihydroxynaphthalene (ϵ_{340} = 2,400).

thalene are produced from *cis*-naphthalene dihydrodiol (Fig. 9).

DISCUSSION

The bacterial oxidation of aromatic substrates that do not contain a hydroxyl group often proceeds through *cis*-dihydrodiol intermediates (11). At this time only the dehydrogenases that catalyze the oxidation of the *cis*-dihydrodiols formed from benzene (2) and benzoic acid (32) have been purified. These enzymes were obtained from a *Pseudomonas* species and *Alcaligenes eutrophus*, respectively. In addition, a diol dehydrogenase has been partially purified from rabbit liver (3). The enzymes obtained from bacteria are similar with regard to their specificity for *cis*-dihydrodiols and their absolute requirement for NAD^+ as an electron acceptor. The diol dehydrogenase from rabbit liver utilizes $NADP^+$ as an electron acceptor, and at high substrate concentrations some activity is also observed with NAD^+. The rabbit liver enzyme was not tested for activity with *cis*-dihydrodiols. However, recent evidence suggests that rabbit liver does contain an enzyme that slowly oxidizes *cis*-benzene dihydrodiol (22). Whether or not more than one diol dehydrogenase exists in mammalian liver remains to be determined.

The molecular weight and subunit size of the *cis*-dihydrodiol dehydrogenases from *P. putida* (102,000 and 25,000) and *A. eutrophus* (94,600 and 24,000) are similar and contrast with the *cis*-benzene dihydrodiol dehydrogenase from a *Pseudomonas* species (440,000 and 110,000). It is noteworthy that the latter enzyme contains loosely bound ferrous ions which are essential for full enzymatic activity. The dioxygenase that oxidizes benzene to *cis*-benzene dihydrodiol also requires ferrous ions for activity (15; P. J. Geary, personal communication). That a coupled dioxygenase-diol dehydrogenase complex exists in the intact organism is a possibility. A coupled monooxygenase-epoxide hydrase complex has been demonstrated in mammalian microsomes (29).

The *cis*-naphthalene dihydrodiol dehydrogenase described in this report is the only enzyme that has been examined for its ability to oxidize a variety of *cis*-dihydrodiol substrates. The enzyme is specific for the (+)-isomer of *cis*-naphthalene dihydrodiol. In data as yet unpublished, we have shown that the enzyme will resolve a racemic mixture of synthetic *cis*-naphthalene dihydrodiol. Other microbiologically

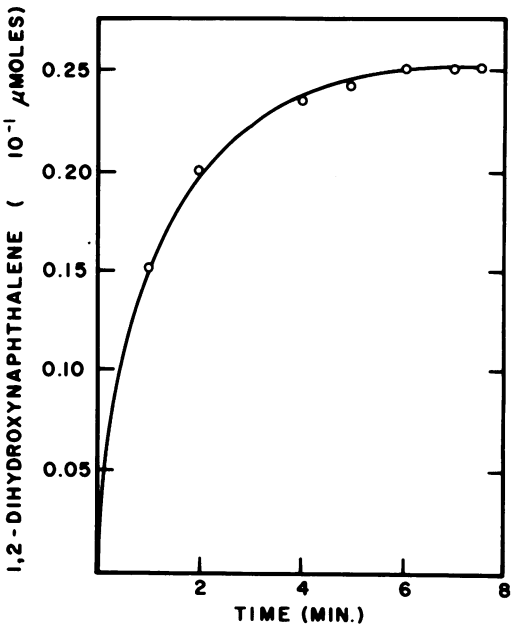

FIG. 9. *Enzymatic formation of 1,2-dihydroxynaphthalene. The reaction mixture contained in a final volume of 3.0 ml: purified enzyme (100 μg of protein); NAD^+, 8.0 μmol; cis-naphthalene dihydrodiol, 0.025 μmol; and KH_2PO_4 buffer (pH 7.0), 275 μmol. A reference cuvette contained all the above components except cis-naphthalene dihydrodiol. Each cuvette was made anaerobic by alternately evacuating and flushing with nitrogen for 20 min. The reaction was initiated by the addition of substrate from the side arm of the cuvette. The formation of 1,2-dihydroxynaphthalene was followed by the increase in absorbance at 231 nm. The amount of 1,2-dihydroxynaphthalene formed was calculated using ϵ_{231} = 41,400.*

produced *cis*-dihydrodiols that serve as substrates for the enzyme are *cis*-anthracene dihydrodiol, *cis*-phenanthrene dihydrodiol *cis*-biphenyl dihydrodiol, *cis*-ethylbenzene dihydrodiol *cis*-toluene dihydrodiol, and *cis*-benzene dihydrodiol. It is interesting to note that none of the K-region *cis*-dihydrodiols of the carcinogenic polycyclic hydrocarbons (6) served as substrates.

The information obtained from bacterial *cis*-dihydrodiol dehydrogenases illustrates the differences between the mechanisms of catechol formation in eukaryotic and prokaryotic cells. Many aromatic compounds are oxidized by eukaryotic cells to arene oxides (6). This reaction occurs with the incorporation of one atom of molecular oxygen into the substrate. Arene oxides then undergo the enzymatic addition of water, by the enzyme epoxide hydrase, to form *trans*-dihydrodiols (28). Further oxidation of *trans*-dihydrodiols by an $NADP^+$-requiring dehydrogenase results in the formation of catechols. In contrast, many bacteria incorporate both atoms of molecular oxygen into non-hydroxylated aromatic substrates, and *cis*-dihydrodiols are the first detectable products (11). Catechols are then formed in an NAD^+-dependent dehydrogenation reaction.

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