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Bacterial *cis*-Dihydrodiol Dehydrogenases: Comparison of Physicochemical and Immunological Properties

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Cells of *Pseudomonas putida* NP, *Pseudomonas* species (NCIB 9816), and a *Nocardia* species, after growth on naphthalene as sole source of carbon and energy, contain a nicotinamide adenine dinucleotide (NAD^+)-dependent enzyme that oxidizes *cis*-dihydrodiols of mono- and polycyclic aromatic compounds. Similarly, cells of a strain of *P. putida* biotype A, when grown either on toluene or benzene vapors, were found to contain a dehydrogenase that oxidized dihydrodiols of aromatic hydrocarbons with *cis* stereochemistry and required NAD^+ as an electron acceptor. In all these cases, no enzymatic activity was detected when *trans*-naphthalene dihydrodiol was used as a substrate. Purified *cis*-naphthalene dihydrodiol dehydrogenase was injected into rabbits to obtain antibodies. Physicochemical and immunological properties of *cis*-dihydrodiol:NAD^+ oxidoreductases from four different organisms were examined. Kinetic analysis showed that, in all the cases, enzymes exhibited higher affinity for *cis*-dihydrodiols than for NAD^+ and had pH optima between 8.8 and 9.0, except in the case of the enzyme from *Nocardia* sp., which showed maximum activity at pH 8.4. Molecular-weight determination of the dehydrogenases from the four different organisms by gel filtration on a Sephadex G-200 column gave values ranging from 92,000 for the enzyme from *Nocardia* sp. to 160,000 for that from *P. putida* biotype A. All the dehydrogenases, except the one from *Nocardia* sp., exhibited immunological cross-reaction with the antibodies prepared against the enzyme purified from *P. putida* NP.

Both bacteria and mammals oxidize naphthalene to a common intermediate, 1,2-dihydroxynaphthalene (7). However, the pathways pursued by these two systems to derive this common metabolite are different. Bacteria carry out double hydroxylation reactions catalyzed by a dioxygenase system that incorporates two atoms of a molecular oxygen into the substrate to form (+)-*cis*-1(R),2(S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (16, 18). Similarly, the bacterial oxidation of benzene (8) and benzoic acid (30, 32, 35) has been shown to involve dioxygenases. Although dioxygenases from benzene- (4), benzoic acid- (35), and toluene- (D. Gibson, W. K. Yeh, and E. Liu, unpublished data) oxidizing bacteria have been resolved into their components, the exact mechanism by which oxygen is fixed into aromatic substrates is not clear. Nevertheless, it is interesting to note that the oxidation of several mono- and polycyclic hydrocarbons by bacteria leads to the formation of dihydrodiols with *cis* stereochemistry (7). To date, the absolute stereochemistry of dihydrodiols formed from toluene (10, 23, 36), naphthalene (16, 18), 3-methylcyclohexene (37), and anthracene (1) dihydrodiols has been determined. Dihydrodiols with *cis* stereochemistry have also been reported during bacterial oxidations of benzene (8, 11, 15), *p*-chlorotoluene (12), biphenyl (13), ethylbenzene (9), and benzoic acid (32).

In mammals, however, the mechanism of single hydroxylation of aromatic compounds involves a monooxygenase system that catalyzes incorporation of one atom of oxygen, forming an arene oxide (6, 17). Subsequent addition of water catalyzed by the enzyme epoxide hydrase yields *trans*-dihydrodiols (19, 20, 27, 28).

The oxidation of *trans*-dihydrodiols formed during the detoxification of aromatic compounds by mammalian liver microsomes and of *cis*-dihydrodiols produced during bacterial oxidation of similar compounds requires dehydrogenases, which apparently differ in their specificity toward substrates. The enzyme from mammalian microsomal preparations requires nicotinamide adenine dinucleotide phosphate ($NADP^+$) as an electron acceptor and oxidizes *cis*-3,5-cyclohexadiene-1,2-diol (*cis*-benzene dihydrodiol) and *trans*-3,5-cyclohexadiene-1,2-diol

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(*trans*-benzene dihydrodiol) (21). This enzyme from mammalian liver has never been purified to homogeneity. Ayengar et al. (5) partially purified an $NADP^+$-linked dehydrogenase from a rabbit liver that oxidized *trans*-benzene dihydrodiol to 1,2-dihydroxybenzene (pyrocatechol). Recently, enzymes that oxidize the *cis*-dihydrodiols of benzene (3), benzoic acid (31), naphthalene (29), and toluene (D. T. Gibson and J. E. Rogers, unpublished data) have been purified and characterized. In this report an examination of dihydrodiol dehydrogenases from four different bacterial strains is described. The physicochemical and immunological properties of the dehydrogenases from *Pseudomonas putida* NP, *Pseudomonas* sp. NCIB 9816, *Nocardia* sp., and *P. putida* biotype A are compared here for the first time.

MATERIALS AND METHODS

Chemicals. The procedures for the preparation of *cis*-3,5-cyclohexadiene-1,2-diol (*cis*-benzene dihydrodiol), *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (*cis*-toluene dihydrodiol), *cis*-3-phenyl-3,5-cyclohexadiene-1,2-diol (*cis*-biphenyl dihydrodiol), *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (*cis*-phenanthrene dihydrodiol), *cis*-1,2-dihydroxy-1,2-dihydroanthracene (*cis*-anthracene dihydrodiol), and *cis*-naphthalene dihydrodiol have been reported previously (29). *trans*-1,2-Dihydroxy-1,2-dihydronaphthalene (*trans*-naphthalene dihydrodiol) was a generous gift from D. M. Jerina, National Institutes of Health, Bethesda, Md. NAD^+ and $NADP^+$ were products of Sigma Chemical Co., St. Louis, Mo. Sephadex G-200 was from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). All other materials were of the highest purity commercially available.

Organisms. *P. putida* NP was isolated from soil by enrichment culture on naphthalene as a sole source of carbon and energy. *P. putida* biotype A was previously described as biotype B (12); this organism has now been reclassified as biotype A (P. J. Chapman, personal communication). *Pseudomonas* sp. NCIB 9816 was kindly provided by W. C. Evans, University of North Wales, Great Britain. A pigmented organism was isolated by enrichment culture on naphthalene as sole source of carbon and energy. The isolated organism was gram positive, non-acid fast, and formed substrate and aerial mycelia. On the basis of these limited observations, it has been tentatively assigned to the genus *Nocardia*.

The differences between the three *Pseudomonas* strains are seen in their ability to use aromatic hydrocarbons as growth substrates. *P. putida* NP and *Pseudomonas* sp. NCIB 9816 grow well with naphthalene, but not toluene, as the sole source of carbon and energy. The dihydrodiol dehydrogenase from the former organism is specific for NAD^+ as an electron acceptor. In contrast, crude cell extracts of strain NCIB 9816 can use NAD^+ and $NADP^+$ as electron acceptors. *P. putida* biotype A grows well with benzene, toluene, or ethylbenzene as the sole source of carbon and energy. Aromatic hydrocarbons containing more than one benzene ring are ineffective as growth substrates.

Growth of organisms. The mineral salt medium used was that of Stanier (33). *P. putida* NP, *Pseudomonas* sp. NCIB 9816, and *Nocardia* sp. were grown in mineral salts medium (pH 6.9) containing 0.2% naphthalene. *P. putida* biotype A was grown in mineral salts medium in the presence of either toluene or benzene vapors as described by Gibson et al. (12). Small-scale liquid cultures were grown in 2-liter Erlenmeyer flasks containing 500 ml of medium. Incubation was carried out at 30°C on a reciprocating shaker. Large-scale cultures (10 to 12 liters) were grown under forced aeration in a fermenter (New Brunswick Microferm). Cells were harvested by centrifugation in a Sorvall centrifuge run at 13,000 × *g* for 5 min and subsequently washed with 20 mM KH_2PO_4 buffer (pH 7.2). Washed cells were frozen and stored at −10°C until used.

Preparation of cell extracts. Washed cells were suspended in 20 mM KH_2PO_4 buffer, pH 7.2, containing 20 mM 2-mercaptoethanol (1.0 g [wet weight] per 3.0 ml of buffer), and were disrupted using an ultrasonic disintegrator (Biosonik III). Unbroken cells and cell debris were removed by a low-speed (13,000 × *g*) centrifugation for 15 min in a Sorvall centrifuge. The clear supernatant solution was centrifuged at 105,000 × *g* for 60 min in an analytical ultracentrifuge (Beckman model L). The supernatant solution was decanted and was designated as a crude extract.

Protein in cell extracts was estimated by the Biuret method (14) or the procedure of Lowry et al. (25).

Antisera. Antisera to the purified *cis*-naphthalene dihydrodiol dehydrogenase and the crude cell extracts from *P. putida* NP were prepared as described previously (29). The gamma globulin (IgG) fraction from the rabbit sera was prepared by the method of Kekwick (22) and Levy and Sober (24).

Enzyme assays. Enzyme activity was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm. Reaction mixtures of 3.0 ml contained (in μmol): NAD^+, 8.0; *cis*-dihydrodiol, 1.0; crude extracts (0.4 to 3.8 mg of protein) and KH_2PO_4 buffer (pH 7.0), 270. The reaction was initiated by addition of a substrate (*cis*-dihydrodiol). Reactions were performed at pH 7.0, since at higher pH values the autooxidation of the reaction product interfered with the enzyme assay. The substrate concentrations used in these experiments were in saturating amounts. Tangents drawn to the recorded curves obtained during the first 15 s of the reaction were used to calculate the initial velocity.

One enzyme unit 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.

Immunodiffusion. The Ouchterlony double-diffusion reactions were carried out as described by Stollar and Levine (34). The concentration of the IgG fraction of rabbit anti-dihydrodiol dehydrogenase was adjusted to 10 mg per ml. The concentration of antigens (cell extracts) that gave sharpest precipitin bands was determined in preliminary tests. All re-

actions were evaluated after incubation for 24 h in a humid atmosphere at room temperature. Pictures of the precipitin bands were taken at the end of the incubation period.

Quantitative precipitin tests. The method of Maurer (26) was employed to estimate the amount of antibody precipitated by various antigens. To a fixed volume, 0.2 ml, of IgG fraction of anti-naphthalene dihydrodiol dehydrogenase (10 mg/ml) was added 0.2 ml of various dilutions of antigens, that is, cell extracts. The mixtures were incubated at 37°C for 1 h followed by an overnight incubation at 4°C. The precipitate formed by antigen-antibody reaction was collected by centrifugation, washed three times in physiological saline, and dissolved in 1.0 ml of 0.02 N NaOH. The dissolved protein was estimated spectrophotometrically at 280 nm.

Inhibition studies. To evaluate the percentage of inhibition of *cis*-dihydrodiol dehydrogenases in cell extracts, varying concentrations of IgG of antienzyme were incubated with cell extracts in a reaction mixture containing the reagents used in a standard enzyme assay as described above. An equivalent amount of IgG fraction of serum from an unimmunized rabbit was preincubated in reaction mixtures as a control. The amount of NAD^+ reduced in the control experiment was compared with that reduced in the presence of antienzyme antibody to appraise the percentage of inhibition.

Molecular-weight determination. The molecular weights of *cis*-dihydrodiol dehydrogenases in crude cell extracts were calculated by the gel filtration technique of Andrews (2) using a calibrated Sephadex G-200 column.

RESULTS

Serological properties of *cis*-dihydrodiol dehydrogenases. Preliminary tests showed that the amount of antisera (in milligrams of protein) required to bring about maximum inhibition of the enzyme activity was reduced by 50% by purifying the IgG fraction of the antiserum (Table 1). The data in Table 1 also indicate that the IgG preparation is about six times as active as the whole-antiserum preparation. The percentage of inhibition of enzyme activity by IgG depended on the specific activity and concentration of protein in a reaction mixture. Hence, to standardize the conditions, the concentration of protein in cell extracts of different organisms was adjusted such that the uninhibited rate of reaction was about the same. The time of preincubation of the enzyme with antiserum had no effect on the percentage of inhibition of the enzyme activity.

Table 2 summarizes the effects of antienzyme antibody on the activity of dehydrogenases in crude extracts of the four bacterial species. The antisera against the enzyme from *P. putida* NP failed to inhibit the enzyme activity in cell extracts of *P. putida* biotype A grown either on toluene or benzene and of *Nocardia* sp. grown on naphthalene as sole source of carbon and energy.

It must be noted that although the anti-*P. putida* NP antiserum did not inhibit the enzyme activity in crude cell extracts of *P. putida* biotype A grown either on toluene or benzene, the latter enzyme reacted with the antibodies to

TABLE 1. *Inhibition of cis-dihydrodiol dehydrogenase activity by antisera and by IgG prepared against the purified enzyme from P. putida NP*[a]

Antisera added (mg/assay)	% Inhibition	IgG added (mg/assay)	% Inhibition
0.0	0	0.00	0
2.0	53	0.25	39
4.0	64	0.50	60
6.0	73	0.75	68
8.0	80	1.00	74
10.0	86	1.50	80
15.0	93	2.00	84
		2.50	86
		3.00	87
		4.00	88
		5.00	90
		8.00	92

[a] Enzyme activity in the presence and absence of antisera was measured spectrophotometrically as described in Materials and Methods. The uninhibited rate of reaction was 0.42 optical density units per min at 340 nm. Each reaction mixture contained 24 μg of the purified enzyme.

TABLE 2. *Inhibition of cis-dihydrodiol dehydrogenase activity in cell extracts of different bacteria*[a]

IgG added (mg/assay)	% Inhibition				
	Source of dehydrogenase[b]				
	NP	NCIB	NOC	A-T	A-B
0.45	35	25	0	0	0
0.90	81	44	0	0	0
1.80	86	67	0	0	0
2.70	89	77	0	0	0
3.60	90	79	0	0	0
5.40	91	82	0	0	0
7.20	91	85	0	0	0

[a] Inhibition studies were made by the procedure described in Materials and Methods. Crude cell extracts were diluted to give uninhibited rates of reactions of about 0.21 optical density units per min at 340 nm.

[b] Crude extracts from: *P. putida* strain NP (NP), *Pseudomonas* sp. NCIB 9816 (NCIB), *Nocardia* sp. (NOC), *P. putida* biotype A grown on toluene (A-T), and *P. putida* biotype A grown on benzene (A-B) were used to obtain the above data.

TABLE 3. *Precipitin tests using IgG prepared against the purified cis-dihydrodiol dehydrogenase from P. putida NP*[a]

Antigen (mg/tube)	Absorption at 280 nm with source of antigen:[b]				
	NP	NCIB	NOC	A-T	A-B
5.0	0.20	0.23	0.0	0.12	0.10
2.5	0.13	0.18	0.0	0.07	0.06
1.25	0.08	0.13	0.0	0.01	0.04
1.625	0.05	ND[c]	0.0	0.01	0.03
0.313	0.04	0.08	0.0	0.01	0.02
0.156	0.03	0.05	0.0	0.0	0.01
0.078	0.03	0.0	0.0	0.0	0.01
0.039	ND	0.0	0.0	0.0	0.0
0.0195	0.01	0.0	0.0	0.0	0.0
0.0098	0.0	0.0	0.0	0.0	0.0

[a] Precipitin tests were carried out by the procedure described in Materials and Methods. The concentration of IgG was kept constant (2.0 mg) in all the tubes, and the concentrations of antigens in each tube were as shown in the table.

[b] Cell extracts of *P. putida* strain NP (NP), *Pseudomonas* sp. NCIB 9816 (NCIB), *Nocardia* sp. (NOC), *P. putida* biotype A grown on toluene (A-T), and *P. putida* biotype A grown on benzene (A-B).

[c] ND, Not determined.

give a precipitate (Table 3). The antigen from *Nocardia* sp. formed no detectable amounts of precipitate. The maximum precipitate formed by the antigens from *P. putida* biotype A grown on toluene and benzene is 50% of that formed by the antigens from *P. putida* NP and *Pseudomonas* sp. NCIB 9816.

The serological cross-reactions of the crude extracts were examined by qualitative gel-diffusion tests (Fig. 1). As illustrated in Fig. 1A, anti-*P. putida* NP serum gave precipitin bands with the homologous antigen and with heterologous antigens from *Pseudomonas* sp. NCIB 9816 and *P. putida* biotype A. However, no precipitin bands were detected in the case of the antigen from *Nocardia* sp. In the case of *P. putida* NP and *Pseudomonas* sp. NCIB 9816, single precipitin bands were obtained that were very sharp, whereas the bands obtained with antigens from *P. putida* biotype A were diffuse. These results indicate that the enzymes from *P. putida* NP and *Pseudomonas* sp. NCIB 9816 possess antigenic determinants common to both organisms. A spur formation was observed (Fig. 1A) when extracts of these organisms were incubated in adjacent wells. This suggests that *P. putida* NP possesses one or more different antigenic determinants that are not present in cell extracts of *Pseudomonas* sp. NCIB 9816. Similarly, when cell extracts of either toluene- or benzene-grown *P. putida* biotype A were incubated in a well adjacent to a well containing extracts of *P. putida* NP, a spur was formed (Fig. 1B and C). This indicates that the enzyme from *P. putida* NP contains an antigenic determinant that is different from that in the enzyme from *P. putida* biotype A.

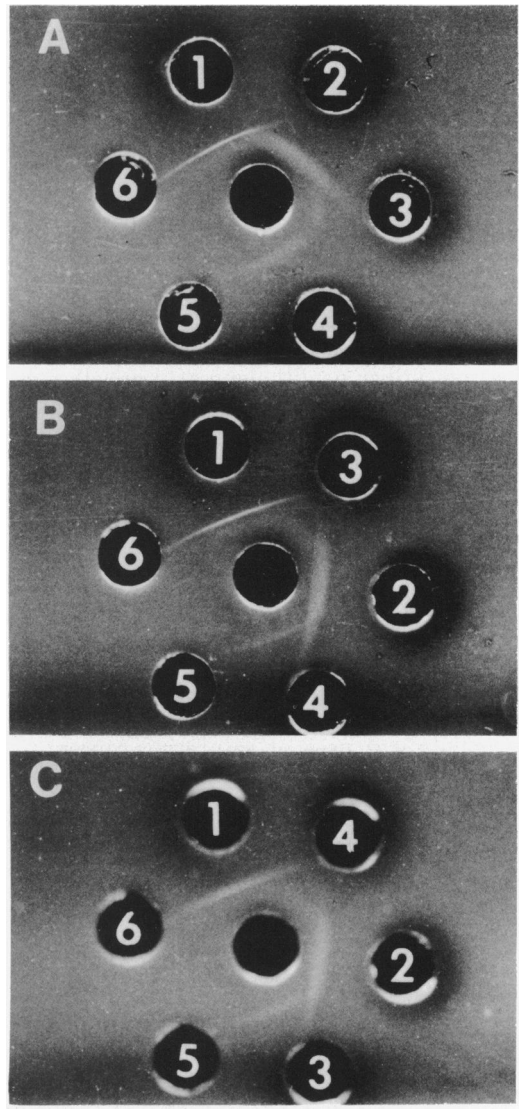

FIG. 1. *Immunodiffusion studies showing cross-reactions between cis-dihydrodiol dehydrogenases of various microorganisms. The center well contained immune gamma globulin prepared against the purified cis-dihydrodiol dehydrogenases from P. putida NP. The other wells contained crude cell extracts of (1) P. putida NP, (2) Pseudomonas sp. NCIB 9816, (3) P. putida biotype A, grown on toluene, (4) P. putida biotype A grown on benzene, (5) Nocardia sp., and (6) none. The cell extracts were diluted to contain 10 mg of protein per ml. The organisms were grown as described in Materials and Methods.*

Physicochemical properties of bacterial *cis*-dihydrodiol dehydrogenases. (i) Substrate specificity. The enzymatic activities of dihydrodiol dehydrogenases in the presence of various dihydrodiols are presented in Table 4. It is interesting to note that none of bacterial dehydrogenases exhibited any detectable activity in the presence of *trans*-naphthalene dihydrodiol, a metabolite produced during naphthalene oxidation in mammals. The enzyme from *P. putida* biotype A grown either on toluene or benzene displayed higher specific activity in the presence of all the *cis*-dihydrodiols tested. A note should be made of the fact that this organism does not utilize naphthalene as a growth substrate and yet oxidizes *cis*-naphthalene dihydrodiol at a rate much higher than that observed in the case of the enzymes from the strains that were grown on naphthalene as a growth substrate. The cell extracts of naphthalene-grown cells of *P. putida* NP, *Pseudomonas* sp. NCIB 9816, and *Nocardia* sp. showed very poor activity in the presence of *cis*-benzene dihydrodiol.

(ii) Apparent K_m values for the substrates. The apparent K_m values for *cis*-dihydrodiols and NAD^+ were obtained from Lineweaver-Burk plots. The second substrate in these experiments was used at saturating concentrations. All the dehydrogenases revealed higher affinity for *cis*-naphthalene dihydrodiol than NAD^+ (Table 5). Cell extracts of *Nocardia* sp. grown on naphthalene and of *P. putida* biotype A grown on toluene showed higher affinity for *cis*-naphthalene dihydrodiol than the remaining extracts.

(iii) Optimum pH. The optimum pH for maximum enzyme activity in cell extracts of the four microorganisms lies in a narrow range between 8.8 and 9.0 (Table 6), except in the case of cell extracts of *Nocardia* sp., which showed an optimum pH of 8.4.

Heat stability. In a preliminary test, cell

TABLE 5. *Effects of NAD^+ and cis-naphthalene dihydrodiol concentrations on the activity of cis-dihydrodiol dehydrogenases from different bacteria*

Source of enzyme	K_m[a]	
	NAD ($\times 10^{-4}$ M)	DIOL[b] ($\times 10^{-5}$ M)
P. putida NP	10.2	2.0
Pseudomonas sp. (NCIB 9816)	10.4	2.4
Nocardia sp.	6.5	1.1
P. putida biotype A[c]	7.1	1.6 (2.4)[e]
P. putida biotype A[d]	7.9	3.5 (5.0)[f]

[a] Apparent values for K_m were obtained from Lineweaver-Burk plots.
[b] DIOL, *cis*-naphthalene dihydrodiol.
[c] *P. putida* biotype A grown on toluene.
[d] *P. putida* biotype A grown on benzene.
[e] Michaelis constant (K_m) for *cis*-toluene dihydrodiol.
[f] Michaelis constant (K_m) for *cis*-benzene dihydrodiol.

TABLE 6. *Effect of pH on the activity cis*-dihydrodiol dehydrogenases from different bacteria[a]

Source of enzyme	Optimum pH
P. putida NP[b]	9.0
Pseudomonas sp. NCIB 9816[b]	9.0
P. putida biotype A[c]	8.9
P. putida biotype A[d]	8.8
Nocardia sp.[b]	8.4

[a] Enzyme assays were performed as described in Materials and Methods.
[b] Grown on naphthalene.
[c] Grown on toluene.
[d] Grown on benzene.

TABLE 4. *Substrate specificity of cis-dihydrodiol dehydrogenases from different bacteria*[a]

Substrate	Sp act[b] with source of dehydrogenase:[c]				
	NP	NCIB	NOC	A-T	A-B
cis-Naphthalene dihydrodiol	0.25	0.42	0.19	0.94	0.62
cis-Benzene dihydrodiol	0.05	0.08	0.02	0.72	0.36
cis-Toluene dihydrodiol	0.08	0.56	0.10	0.89	0.62
cis-Anthracene dihydrodiol	0.15	0.73	0.05	0.55	0.25
cis-Phenanthrene dihydrodiol	0.23	0.28	0.04	0.34	0.20
cis-Biphenyl dihydrodiol	0.09	0.26	0.02	0.36	0.18
trans-Naphthalene dihydrodiol	0.0	0.0	0.0	0.0	0.0

[a] The assay mixtures contained (in μmol) in a final volume of 3.0 ml: substrate, 1.0; NAD^+, 7.0; KH_2PO_4 buffer (pH 7.0), 290; and cell extracts, 0.4 to 3.8 mg of protein. Initial velocities were measured as described in Materials and Methods.
[b] Specific activity is defined in Materials and Methods.
[c] Cell extracts of *P. putida* strain NP (NP), *Pseudomonas* sp. (NCIB 9816) (NCIB), *Nocardia* sp. (NOC), *P. putida* biotype A grown on toluene (A-T), and *P. putida* biotype A (A-B) grown on benzene.

extracts of the four organisms were incubated at 60°C. In about 5 min the enzyme activity in all the extracts was totally lost. However, when the same extracts were incubated at 50°C, a gradual decay of enzyme activities was observed, as shown in Fig. 2. The dehydrogenase in the cell extracts of *Pseudomonas* sp. NCIB 9816 and *Nocardia* sp. showed least stability at 50°C. In contrast, the extract of *P. putida* NP retained most of the initial activity at the end of 60-min incubation. However, the same extract, when incubated at 57°C, showed a rapid loss of the enzyme activity, and as much as 65% of the initial activity was lost in 30 min.

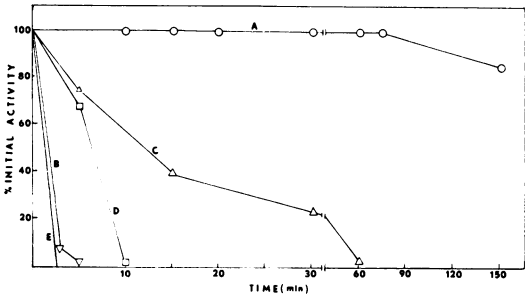

FIG. 2. *Thermal stability of cis-dihydrodiol dehydrogenases from different bacteria. The protein concentration in cell extracts was 10 mg/ml, except in the case of cell extract of Nocardia sp., which contained 4 mg of protein per ml. The cell extracts were incubated at 50°C in a water bath, and the enzymatic activity was measured spectophotometrically as described in Materials and Methods. Cell extracts of P. putida strain NP (A), Pseudomonas sp. (NCIB 9816) (B), P. putida grown on toluene (C), P. putida biotype A grown on benzene (D), and Nocardia sp. (E) were used in this experiment.*

Molecular weight. The determination of molecular weights of *cis*-dihydrodiol dehydrogenases in crude extracts of the four organisms was carried out by gel filtration on a calibrated Sephadex G-200 column. From the results obtained, a standard plot of K_{av} versus the molecular weight was plotted, as shown in Fig. 3. The molecular weights of the dehydrogenases were calculated using this standard curve and are summarized in the legend for Fig. 3.

DISCUSSION

The degradation of several aromatic compounds by soil bacteria proceeds through *cis*-dihydrodiols (7). The dehydrogenases that oxidize the *cis*-dihydrodiols formed from benzene (3), benzoic acid (31), and naphthalene (29) have been purified from a *Pseudomonas* sp., *Alcaligenes eutrophus*, and *P. putida* NP, respectively. The enzyme that oxidizes *cis*-benzoic acid dihydrodiol has also been detected in species of *Pseudomonas*, *Acinetobacter*, and *Azotobacter* (30). It should be noted that the *cis* stereochemistry of this dihydrodiol has not been firmly established. The enzyme purified from *P. putida* NP (16) grown on naphthalene is the only dihydrodiol dehydrogenase that has been tested for its absolute stereospecificity toward *cis*-naphthalene dihydrodiol. It is specific

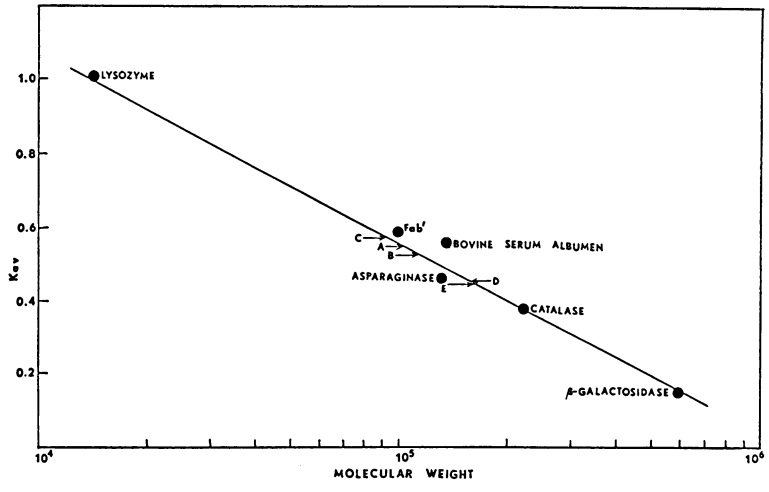

FIG. 3. *Molecular weights of cis-dihydrodiol dehydrogenases from different bacteria as determined by Sephadex G-200 gel filtration. The standard proteins used are shown in the figure. The letters in the graph represent enzymes with molecular weights in parentheses from: A, P. putida strain NP (102,000); B, Pseudomonas sp. NCIB 9816 (112,000); C, Nocardia sp. (92,000); D, P. putida biotype A grown on toluene (155,000); and E, P. putida biotype A grown on benzene (160,000). The enzymatic activity in column effluents was measured by reduction of NAD$^+$ at 340 nm in the presence of cis-naphthalene dihydrodiol.*

for the (+) isomer of *cis*-naphthalene dihydrodiol (16). This is the first report in which dihydrodiol dehydrogenases from four different organisms have been simultaneously tested for their ability to oxidize a variety of *cis*-dihydrodiols. The bacterial dihydrodiol dehydrogenases (3, 29–31) reported in the literature, together with the ones described in this report, utilize *cis*-dihydrodiols as substrates and require NAD^+ as an electron acceptor. Ayengar et al. (5) partially purified a dehydrogenase from rabbit liver which utilized $NADP^+$ as an electron acceptor, and at high substrate concentration some activity was also observed with NAD^+. This enzyme from rabbit liver was not tested for its ability to oxidize *cis*-dihydrodiols. However, recent evidence suggests that the liver enzyme oxidizes *cis*-benzene dihydrodiol in addition to its *trans* isomer (21). It is not known whether there is more than one dihydrodiol dehydrogenase in mammals.

Immunodiffusion experiments did not provide evidence for any serological relationships between the enzyme from *Nocardia* sp. and *P. putida* NP. In contrast, the enzymes from *Pseudomonas* sp. NCIB 9816 and *P. putida* NP show similarity in their immunological properties. The enzymes from these organisms were inhibited and precipitated to a similar extent by IgG prepared against the purified enzyme from *P. putida* NP. Precipitin bands showed a spur (Fig. 1A), indicating that the enzyme from *P. putida* NP contains antigenic determinant(s) not shared by the enzyme from *Pseudomonas* sp. NCIB 9816.

Immunodiffusion tests also revealed a partial immunological relationship between the enzyme from *P. putida* biotype A (grown either on toluene or benzene) and that from *P. putida* NP. This conclusion is based on a weak precipitin band observed when the antigen from the former organism is incubated in a well adjacent to the one containing the antigen from the latter organism (Fig. 1B and C). This observation was further confirmed in a quantitative precipitin test. The IgG prepared against the purified enzyme from *P. putida* NP formed a precipitate with the enzyme from *P. putida* biotype A. However, the same IgG failed to inhibit the enzyme activity in cell extracts of *P. putida* biotype A. This suggests that the enzyme from this organism contains antigenic determinant(s) not including regions of the active site.

In immunodiffusion experiments in which cell extracts of *P. putida* biotype A grown on toluene and benzene were incubated in adjacent wells (Fig. 1A), they formed precipitin bands that were fused (lines of complete identity), indicating a close homology of the two enzymes. The two enzymes probably represent closely similar or identical proteins. This would be possible if the mechanism of induction of the enzyme synthesis in benzene- and toluene-grown cells is similar. The enzymes from *P. putida* biotype A grown separately on two different substrates, toluene and benzene, share the following characteristics: neither enzyme is inhibited by IgG but both are precipitated to a similar extent by the same IgG; both give weak precipitin bands with IgG; and both have similar molecular weights and optimum pH values. However, the enzyme from the toluene-grown cells has greater heat stability at 50°C and showed higher specific activities than that from benzene-grown cells. These findings indicate that the enzymes from *P. putida* biotype A grown on benzene and toluene indeed may represent homologous proteins. The dissimilarities in their properties may represent minor differences in their primary structures and therefore in their protein conformations.

The following properties are common to the enzymes from *Pseudomonas* sp. NCIB 9816 and *P. putida* NP: both the enzymes are similar in their molecular size, have similar pH optima, possess similar affinity for *cis*-naphthalene dihydrodiol, show strong cross-reactions with IgG, and are inhibited by IgG to a similar degree. Nevertheless, the enzyme from *P. putida* NP is much more stable at 50°C than the one from *Pseudomonas* sp. NCIB 9816. Also, the enzyme from the former organism showed a spur formation in the Ouchterlony double-diffusion tests, indicating the presence of antigenic determinant(s) not shared with the enzyme from the latter organism. The dehydrogenase from *Pseudomonas* sp. NCIB 9816 displayed higher activity in the presence of all the dihydrodiols tested. Thus, the resemblance in the immunological and the biochemical properties of the enzymes from these two organisms suggests a structural homology of the proteins. The differences in their properties may reflect minor alterations in their tertiary structures.

One of the distinguishing features of the enzyme from *Pseudomonas* sp. (3) is its molecular weight (440,000). The molecular weight of a similar enzyme from *P. putida* biotype A grown on the same substrate was 160,000. Moreover, the larger enzyme required ferrous ions and glutathione for maximum activity (3). The possibility of metal ions tightly bound to other dehydrogenases described in this report cannot be ruled out.

It is shown in this report that the oxidation of *cis*-dihydrodiols of aromatic compounds by procaryotes involves oxidoreductases that are specific for dihydrodiols with *cis* stereochemistry

and require NAD^+ as an electron acceptor. In contrast, eucaryotes carry dihydrodiol dehydrogenases that require $NADP^+$ as an electron acceptor. More work is needed to show whether there is more than one diol dehydrogenase in eucaryotes. So far, there is only a single report (22) that suggests that mammalian microsomal preparation can oxidize *cis*- as well as *trans*-benzene dihydrodiols.

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