Initial Reactions in the Oxidation of Naphthalene by *Pseudomonas putida*†

A. M. Jeffrey, H. J. C. Yeh, D. M. Jerina,*
T. R. Patel, J. F. Davey, and D. T. Gibson*

ABSTRACT: A strain of *Pseudomonas putida* that can utilize naphthalene as its sole source of carbon and energy was isolated from soil. A mutant strain of this organism, *P. putida* 119, when grown on glucose in the presence of naphthalene, accumulates optically pure (+)-*cis*-1(*R*),2(*S*)-dihydroxy-1,2-dihydronaphthalene in the culture medium. The cis relative stereochemistry in this molecule was established by nuclear magnetic resonance spectrometry. Radiochemical trapping experiments established that this cis dihydrodiol is an intermediate in the metabolism of naphthalene by *P. fluorescens* (formerly ATCC, 17483), *P. putida* (ATCC, 17484), and a *Pseudomonas* species (NCIB 9816), as well as the parent strain of *P. putida* described in this report. Formation of the cis dihydrodiol is catalyzed by a dioxygenase which requires either NADH or NADPH as an electron donor. A double label procedure is described for determining the origin of oxygen in the cis dihydrodiol under conditions where this metabolite would not normally accumulate. Several aromatic hydrocarbons are oxidized by cell extracts prepared from naphthalene-grown cells of *P. putida*. The cis dihydrodiol is converted to 1,2-dihydroxynaphthalene by an NAD^+-dependent dehydrogenase. This enzyme is specific for the (+) isomer of the dihydrodiol and shows a primary isotope effect when the dihydrodiol is substituted at C-2 with deuterium.

The ability of soil microorganisms to oxidize nonpolar aromatic hydrocarbons has been recognized for many years. Substrates range from benzene to complex, virtually water-insoluble polycyclic hydrocarbons such as benzo[*a*]pyrene (Evans, 1969; Dagley, 1971; Zobell, 1971). The initial oxidative step in the metabolism of many aromatic hydrocarbons by bacteria is the formation of 1,2-dihydrodiols (Gibson, 1971; Treccani, 1974), compounds in which the elements of hydrogen peroxide have been added across one of the aromatic double bonds. Assignment of relative stereochemistry to dihydrodiols formed from naphthalene, phenanthrene, anthracene, and derivatives thereof has been difficult because only very small amounts of the diols accumulate in the incubation medium. Knowledge of the relative stereochemistry of these dihydrodiols has significance in that it relates directly to the mechanism(s) by which the oxygen is introduced into the aromatic ring, a process about which relatively little is known (Jerina, 1973). The present investigation deals with the characterization of the dihydrodiol formed from naphthalene by *Pseudomonas putida* and also some properties of the dioxygenase and the dehydrogenase involved in the initial stages of naphthalene metabolism. Possible mechanisms are discussed and contrasted with related mammalian oxidations.

Materials and Methods

Materials. [$^2H_8$]Naphthalene (98 atom %), $^{18}O_2$ (99.3 atom %), and $^2H_2$ (99.9 atom %) were obtained from Isomet Corporation. [^{14}C]Naphthalene (diluted to 25 mCi/mmol) was obtained from Nuclear-Chicago. The following compounds were prepared as described: 1- and 2-hydroxy-1,2-dihydronaphthalene (Jeffrey and Jerina, 1972); (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene (Booth *et al.*, 1950); (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene (Jeffrey *et al.*, 1974); 1,2-dihydroxynaphthalene (Corner and Young, 1954); *cis*- and *trans*-*o*-formylcinnamaldehyde (periodate oxidation of the cis dihydrodiol, unpublished). [1,4-$^2H_2$]Naphthalene was prepared by deuterolysis of 1,4-dibromonaphthalene in analogy to previously described procedures (Jerina *et al.*, 1971b). The product contained 10% of the molecules with only one deuterium while the remaining 90% were dideuterated. [2,6-$^2H_2$]Naphthalene was obtained from 6-bromo-2-naphthol by prior conversion to a tetrazoyl ether with 5-chloro-1-phenyl-1(*H*)-tetrazole (Musliner and Gates, 1966) followed by exhaustive deuterolysis. The [2,6-$^2H_2$]naphthalene consisted of 3% undeuterated, 21% monodeuterated, and 76% dideuterated species. Incubation of the deuterated naphthalenes with washed cell suspensions of *P. putida* strain 119, grown as described, provided the corresponding deuterated cis dihydrodiols which were isolated by extraction of the medium with ethyl acetate and purification of the extract by tlc. Deuterium contents of the diols corresponded to those in the starting naphthalenes by mass spectrometry. Integration of the nmr spectra of these diols at the C-1 *vs.* the C-2 positions established the extent of deuterium labeling at these sites as 48 and 40% in the respective diols. Completely deuterated diol was obtained from [$^2H_8$]naphthalene in a similar fashion. Details of these incubations and isolations were comparable to those for unlabeled naphthalene described later. Incuba-

† From the National Institute of Arthritis, Metabolism, and Digestive Diseases, The National Institutes of Health, Bethesda, Maryland 20014 (A.M.J., H.J.C.Y., D.M.J.) and the Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712 (T.R.P., J.F.D., and D.T.G.). *Received September 9, 1974.* Studies were supported in part by Grants ES-00537 from the National Institutes of Health, U.S. Public Health Service, and F-440 from the Robert A. Welch Foundation. D.T.G. is a recipient of Career Development Award 1 K04 ES-70088, from the National Institutes of Health, U.S. Public Health Service, and A.M.J. was supported in part by a NATO Postdoctoral fellowship.

tions of rat liver microsomes with naphthalene were as previously described (Jerina *et al.*, 1970) with the exceptions noted.

Other chemicals were commercially available. Thin-layer chromatography was performed on silica gel with fluorescent indicator using either Eastman chromatogram sheets (13181) or Analtech glass plates.

Methods. Optical rotations were measured with a Perkin-Elmer Model 141 automatic polarimeter, while circular dichroism spectra were obtained on a Cary 60. The nmr spectra were recorded at 100 MHz in $CDCl_3$ with a Varian HA-100 instrument. Mass spectra were measured with a Hitachi RMU-7 spectrometer at 70 eV. For isotopically labeled compounds, the ionizing voltage was reduced to 10–15 eV. Radioactivity was measured by liquid scintillation counting using Aquasol from New England Nuclear as scintillation fluid.

ISOLATION AND GROWTH OF MICROORGANISMS. A bacterium, identified as a strain of *Pseudomonas putida* (Stanier *et al.*, 1966), was isolated from soil for its ability to grow on naphthalene as the sole carbon and energy source. A mutant strain, unable to grow on naphthalene as sole source of carbon, was obtained from the wild type organism after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Ornston, 1966). This organism (*P. putida* strain 119) accumulated (+)-dihydrodiol when grown on glucose in the presence of naphthalene. Other *Pseudomonas* species used in this study were *P. fluorescens* (formerly ATCC 17483) and *P. putida* biotype B (ATCC 17484) obtained from the American Type Culture Collection and a *Pseudomonas* species (NCIB 9816), obtained from Professor W. C. Evans, the University College of North Wales, Bangor, United Kingdom. With the exception of the isolation experiment described below, all organisms were grown with forced aeration at 30° in mineral salts medium (Stanier *et al.*, 1966). Naphthalene (0.1%) with or without glucose (0.2%) was added to the medium.

ISOLATION AND IDENTIFICATION OF (+)-*cis*-1(*R*),2(*S*)-DIHYDROXY-1,2-DIHYDRONAPHTHALENE. The metabolite can readily be obtained from preparative scale fermentation. A solution of 300 l. of mineral salts medium contained, per liter, 5 ml of fortifying salt solution (Bauchop and Elsden, 1960), 0.25 g of nitrilotriacetic acid, 1.8 g of glucose, 1.3 g of ammonium sulfate, and 6.4 g of potassium dihydrogen phosphate. The final solution was adjusted to pH 7.0 with sodium hydroxide. After addition of 400 g of naphthalene and a 1% inoculum of *P. putida* strain 119, the mixture was incubated at 25° for 1 day with forced aeration. The cells were removed by centrifugation, and the supernatant solution was concentrated under vacuum. Much of the concentrate was lost due to an electrical power failure. The remaining solution was extracted with ethyl acetate, which on drying (Na_2SO_4) and concentration provided 58 g of crude metabolite estimated to be >95% pure. The material crystallizes from warm ethyl acetate or chloroform as fine needles: mp 115–116°; $[\alpha]^{25}D$ +220 ± 3° (*c* 0.05–0.1, methanol). On the basis of spectral properties and elemental analysis (Jerina *et al.*, 1971a), the metabolite had been assigned as (+)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene.

Rates of dehydration for the cis and trans dihydrodiols (λ_{max} 262 nm, ethanol; ϵ_{cis} 8114; ϵ_{trans} 7068) were measured in aqueous hydrochloric acid at 25°. In separate experiments, 100 μl of a 2.0 mM solution of each diol in ethanol was added to 2.4 ml of hydrochloric acid in a quartz cuvet to produce a final concentration of 2.4 M acid. The first-order disappearance of diol was monitored by a decrease in absorbance at 265 nm. The rates observed for the dehydration of the cis and trans isomers were 2.94×10^{-2} and 6.63×10^{-4} sec^{-1}, respectively. Analysis of the products from larger scale experiments by tlc showed that only naphthols were formed. In each case, the mixture consisted of >95% 1-naphthol.

The cis dihydrodiol was readily converted to an acetonide by stirring a suspension of the diol in a large excess of 2,2-dimethoxypropane (Fieser and Fieser, 1967) containing a drop of concentrated hydrochloric acid. Within 1 hr the diol had dissolved and reaction was complete. After removal of the solvent under reduced pressure, a near quantitative yield of crude acetonide was obtained as an oil which could not be induced to crystallize despite indications from the nmr spectrum and tlc that the material was essentially pure. The acetonide was also prepared by stirring a solution of the diol in acetone with anhydrous, powdered copper sulfate in the dark for 3 weeks.

The absolute stereochemistry of the cis dihydrodiol was established by chemically relating it to the known (−)-2(*S*)-hydroxy-1,2,3,4-tetrahydronaphthalene. A sample of crude dihydrodiol was used without crystallization to avoid possible change in optical purity. Two grams of the dihydrodiol was dissolved in 10 ml of pyridine and 4 ml of acetic anhydride at 0° and left at 0° overnight. Water was added and the product extracted into ether. The crude 1,2-diacetoxy-1,2-dihydronaphthalene, after removing the ether, was dissolved in 20 ml of ethanol and reduced with hydrogen in the presence of 100 mg of 10% Pd on carbon until 1 mol of hydrogen had been absorbed. Catalyst and solvent were removed. The 1,2-diacetoxy-1,2,3,4-tetrahydronaphthalene in 60 ml of acetic acid was reduced with hydrogen and 1 g of 10% Pd on carbon for 24 hr to give a 90% yield of 2-acetoxy-1,2,3,4-tetrahydronaphthalene (Augustine, 1965). The ester was hydrolyzed by dissolving it in 12 ml of ethanol and 8 ml of 4 N NaOH and heating to 100° for 10 min. Water (10 ml) was then added and the pH adjusted to 7 with acetic acid before extracting with 3 × 10 ml of ether to give 1.28 g of 2-hydroxy-1,2,3,4-tetrahydronaphthalene. At each stage 100 mg of product was removed for nmr analysis and further purification by tlc for optical rotation measurements. Results are shown in Table I.

PROCEDURE FOR ESTABLISHING STEREOCHEMISTRY OF DIHYDRODIOLS WITHOUT THE USE OF A BLOCKED MUTANT. Since the wild type bacteria do not readily accumulate dihydrodiols from naphthalene due to subsequent metabolism, a radiochemical trapping procedure was devised to establish stereochemistry. In each assay 250 μg of (+)-cis and 250 μg of (±)-trans dihydrodiol, 0.1 μmol of [1-^{14}C]naphthalene (50 nCi), 0.2 μmol of NADH, and 0.2 mmol of KH_2PO_4 (final pH, 7.0) were incubated at 25° with 100–200 μg of protein (crude cell extract, see later) in a final volume of 2 ml. After 2 min, 2 ml of ether was added to extract the dihydrodiols. Separation of the cis and trans carrier diols was achieved by multiple development (4–5 times) of tlc plates with chloroform. The diols, visualized with ultraviolet light, were extracted from the gel with 1.2 ml of methanol and the amount of diol present was estimated spectrophotometrically. Radioactivity was estimated by counting a 1-ml sample of the methanol solution. Regions of silica gel behind, between, and in front of the diol bands were extracted and counted as well to establish that the radioactivity was localized in the bands containing carrier

diols. Incubations with microsomes, prepared from the livers of Sprague Dawley rats, were essentially as described (Jerina *et al.*, 1970) with addition of carrier diol as above. The microsomal incubations were run with the protein equivalent of 250 mg of liver. Zero time recovery of the diols was 30%. Recovery of the cis diol after incubation with the bacterial systems dropped to 15% while recovery of the trans diol remained unchanged. Conversion of naphthalene to recovered diol was >2% except for the microsomal incubation which was much lower. Radioactivity over blank ranged from 3000 to 10,000 dpm (Table II).

ORIGIN OF OXYGEN ATOMS IN THE *cis*-NAPHTHALENE DIHYRODIOL. The origin of the oxygen atoms in the dihydrodiols produced by *P. putida, strain* 119, and from *P. putida* (ATCC 17484) was determined by use of oxygen-18 gas. A two-compartment reaction flask of 45-ml capacity was employed to keep the bacteria and naphthalene separate while the flask was evacuated and refilled with appropriate gas mixtures. The flask was evacuated and filled with nitrogen twice, reevacuated, and filled with oxygen gas at approximately 1 atm which was enriched with ^{18}O as indicated. The contents of the compartment containing 2 ml of cell suspension (~5%) in 0.1 M potassium phosphate buffer at pH 7.0 was mixed with substrate to initiate reaction. Isotope composition of the oxygen gas was determined on residual oxygen at the end of the incubations. For *P. putida* (ATCC 17484) which did not accumulate significant amounts of the dihydrodiol, 1 mg of [$^2H_8$]*naphthalene*, and 1 mg of unlabeled (+)-cis dihydrodiol were incubated for 4 hr at which point 0.1 mg of diol was present of which 80% was deuterated and thus newly synthesized. The diols were isolated by tlc and the oxygen contents determined by mass spectrometry (Table III).

STUDIES WITH *P. putida* CELL EXTRACTS. (A) Preparation of Cell Extracts. Cells grown with naphthalene were harvested in the late stage of logarithmic growth by centrifugation, washed three times with 0.02 M KH_2PO_4 buffer (pH 7.2), and stored at −15°. Frozen cells were ground to a fine powder in a cold mortar or passed through a Hughe's press. Distilled water (2 ml/g of frozen cells) and deoxyribonuclease (1 mg/g of cells) were added and the mixture allowed to stand at room temperature for 30 min. Cell debris was removed by centrifugation at 10,000 *g* for 15 min and the supernatant solution further centrifuged at 105,000*g* for 1 hr. The clear supernatant solution was taken as a source of crude enzyme. Cell extracts for the other organisms listed in Table II were prepared in a similar fashion.

(B) Assay of Oxygenase and Dehydrogenase Activities. Naphthalene dioxygenase activity in the cell extract was assayed by measuring oxygen consumption with a Clark oxygen electrode or spectrophotometrically by NADH or NADPH oxidation. Details of individual experiments are provided in the figure legends. (+)-*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase activity in the cell extract was assayed by following the first-order production of NADH from NAD^+ at 340 nm. The other product of the reaction, 1,2-dihydroxynaphthalene, rapidly autoxidizes to 1,2-naphthoquinone which interferes with the measurements at 340 nm. Consequently most assays were performed under anaerobic conditions where production of NADH and 1,2-dihydroxynaphthalene was stoichiometric. A correction for the absorption of 1,2-dihydroxynaphthalene at 340 nm (ϵ 2400) was applied where necessary. Cells of *P. putida* strain 119, when grown on glucose in the presence of naphthalene, contained no detectable dehydrogenase activity. Cells of *P. putida* wild type when grown on either glucose or succinate contain no detectable oxygenase or dehydrogenase activity.

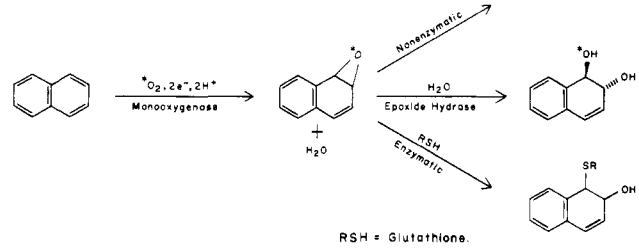
(C) Product Identification. Naphthalene (5.0 μmol) was oxidized, in the presence of NADPH (10 μmol), as described in Figure 3. At the end of the reaction, the mixture was extracted with 2 vol of ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and the solvent removed under reduced pressure. The residue was dissolved in 0.1 ml of ethyl acetate and 10-μl samples were separated by tlc (chloroform–acetone, 4:1). A single ultraviolet absorbing component was present (R_F 0.25) which was indistinguishable from the cis dihydrodiol isolated from cultures of the mutant *P. putida* strain 119 when grown in the presence of naphthalene.

NAD$^+$-DEPENDENT CIS DIHYDRODIOL DEHYDROGENASE. The enzyme activity in cell extracts of *P. putida* which converts *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and NAD^+ into 1,2-dihydroxynaphthalene and NADH has been purified to homogeneity (Patel and Gibson, 1974), and was used in the experiments described (Tables V and VI).

Results and Discussion

1,2-Dihydrodiols have been known as intermediates in the metabolism of aromatic hydrocarbons since 1936 when Boyland and Levi first described *trans*-1,2-dihydroxy-1,2-dihydroanthracene as a metabolite formed from anthracene by mammals. As shown in Scheme I for the mammalian oxidation of naphthalene, the hydrocarbon is first converted to an arene oxide by the cytochrome P-450 monooxygenase system (Jerina *et al.*, 1970; Oesch *et al.*, 1972). Nonenzymatic isomerization to naphthols, reaction with cellular nucleophiles such as glutathione, and enzymatic hydration to *trans*-1,2-dihydroxy-1,2-dihydronaphthalene are the principal reactions of the oxide. The dihydrodiol is subject to further oxidation to a catechol (Ayengar *et al.*, 1959; Jerina *et al.*, 1970). The role of arene oxides in metabolism has been the subject of recent reviews (Daly *et al.*, 1972; Jerina and Daly, 1974).

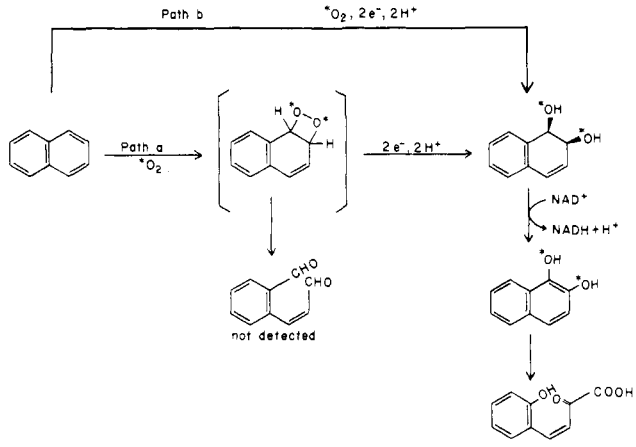
SCHEME I: Oxidation of Naphthalene by Mammals.



The uniform occurrence of trans dihydrodiols as metabolites in mammals initially suggested that bacteria may also oxidize aromatic hydrocarbons through trans dihydrodiols. Bacteria were reported to oxidize naphthalene (Walker and Wiltshire, 1953) and substituted naphthalenes (Canonica *et al.*, 1957) through trans dihydrodiols. A dihydrodiol was also isolated from bacterial cultures that oxidized phenanthrene and indirect evidence suggested the formation of a dihydrodiol during the bacterial oxidation of anthracene (Colla *et al.*, 1960). Since in many instances the microbially produced dihydrodiols do not significantly accumulate in the culture medium, due to subsequent metabolism, only small samples were available for study, and rigorous assign-

ment of stereochemistry was not possible. In sharp contrast to these early studies, recent investigations on the bacterial metabolism of benzene and substituted benzenes have established that these compounds are oxidized through cis dihydrodiols. The latter compounds are formed by the action of a dioxygenase (Gibson, 1971) as illustrated in Scheme II.

SCHEME II: Possible Metabolic Pathways for Naphthalene Oxidation by Bacteria.



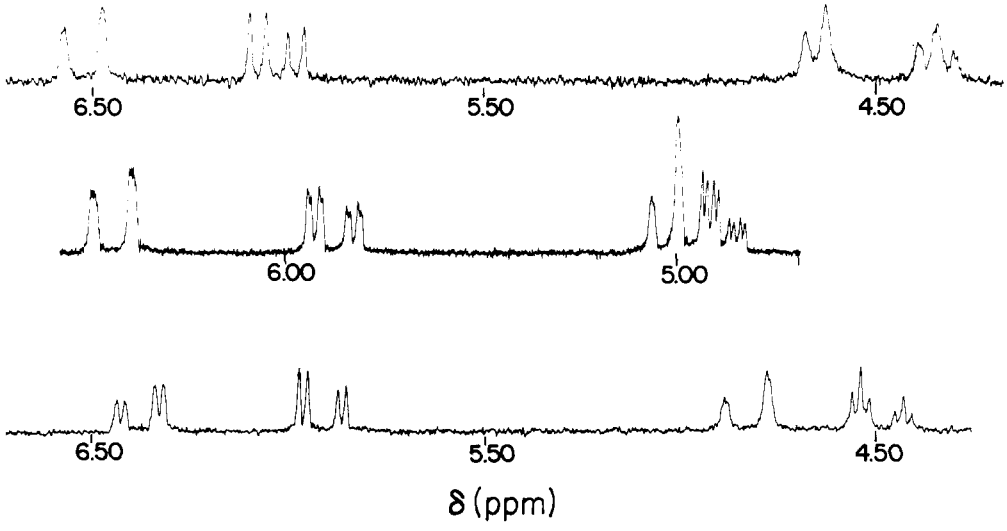
In order to clarify the situation with regard to the bacterial oxidation of naphthalene and other polycyclic molecules, a strain of *P. putida* was isolated from soil by selective enrichment culture with naphthalene as the sole source of carbon and energy. This bacterium is similar to related organisms (Griffiths and Evans, 1965) in that salicylic acid as well as minor amounts of dihydrodiol can be detected in the culture medium during growth with naphthalene. Mutation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulted in isolation of an organism, *P. putida* strain 119, which no longer grew on naphthalene alone. When this organism was grown on glucose, in the presence of naphthalene, the hydrocarbon was converted in 60% yield to a compound assigned as (+)-*cis*-1(*R*),2(*S*)-dihydroxy-1,2-dihydronaphthalene (Jerina *et al.*, 1971a). The same cis dihydrodiol was subsequently identified as an intermediate in the oxidation of naphthalene by *Pseudomonas* sp. NCIB 9816 (Catterall *et al.*, 1971).

To gain insight into the mechanism by which bacteria activate molecular oxygen and convert naphthalene into a dihydrodiol, which is the primary metabolite, it was essential to establish the relative stereochemistry of the hydroxyl groups in this molecule. If the cis stereochemistry pertains, and if both atoms of oxygen originate from the same molecule of molecular oxygen, a 1,2-dioxetane (Scheme II, path a) would present itself as a highly attractive intermediate in the reaction. Alternatively, a direct and stereospecific introduction of the two oxygen atoms (Scheme II, path b) must also be considered. At the outset of this study, the dihydrodiols from mammalian metabolism of naphthalene and higher polycyclic systems were thought to be trans based on considerable indirect evidence. However, unequivocal proof even for this had not been forthcoming.

The overall features of the nmr spectrum (Figure 1) of the bacterial dihydrodiol from naphthalene were such that a 1,2-dihydroxy-1,2-dihydronaphthalene was the only compatible structure. While the bacterial dihydrodiol readily formed an acetonide either with 2,2-dimethoxypropane and a trace of acid or with acetone and anhydrous copper sulfate, the synthetic trans dihydrodiol only produced naphthols. The rate of dehydration of the bacterial dihydrodiol with acid was 44 times faster than the trans isomer and 52 times slower than *cis*-1,4-dihydroxy-1,4-dihydronaphthalene (Jeffrey *et al.*, 1974). The formation of an acetonide and the higher rate of dehydration are consistent with cis stereochemistry in the bacterial dihydrodiol.

Detailed analysis of the nmr spectra of the bacterial and synthetic trans dihydrodiols (Figure 1) provided a definitive means of assigning relative stereochemistry. The relatively small three-bond $^3J_{1-2}$ coupling constant of 5.1 Hz for the carbinol hydrogens in the spectrum of the bacterial dihydrodiol compared with 10.1 Hz for the same hydrogens in the trans isomer is consistent with cis stereochemistry in the bacterial isomer (Karplus, 1963). However, great care must be exercised in drawing conclusions about stereochemistry based on the Karplus relationship, especially when the vicinal coupling constants involve carbon atoms bearing electronegative substituents such as hydroxyl groups (Jackman and Sternhill, 1969). For this reason, an alternate procedure to comparison of the $^3J_{1-2}$ for the two diols was sought for assigning stereochemistry. Inspection of Dreiding stereomodels for dihedral bond angles in the two extreme conformations of the two diols and use of the Karplus relationships as described by Becker (1969) allow prediction of the expected signs and magnitudes for $^3J_{1-2}$, $^3J_{2-3}$, and $^4J_{2-4}$ for the four hydrogens of the dihydro aromatic rings in the two diols. Quite good agreement between the calculated and observed couplings is found (Figure 1) if the trans dihydrodiol is assumed to exist mainly as the conformer with the hydroxyl groups occupying quasiequatorial positions. Presumably any unfavorable steric interaction between the hydroxyl group at C-1 and the peri ring hydrogen at C-8 is overcome by internal hydrogen bonding. The sign of J_{2-4} must be negative in this conformation. A standard spin-tickling experiment (Bovey, 1969) was employed to prove the sign of J_{2-4} to be opposite to the other signs in this four-spin system (H_1 decoupled) and therefore negative. Since stereochemical arguments based on coupling constants are dependent on assignment of conformation, two further experiments were conducted to confirm that the hydroxyl groups in the trans isomer reside preponderantly in the quasiequatorial positions. The value of J_{1-2} for the trans isomer changes dramatically from 10.1 to 6.0 Hz on preparing the diacetate as would be expected since the bulky acetoxy groups will tend to occupy quasiaxial positions in the ester. Furthermore, the value of J_{1-2} increases and J_{2-3} decreases on cooling an acetone solution of the diol to $-70°$ as anticipated for an increase in the population of the state in which both hydroxyl groups are quasiequatorial. At $-70°$ interconversion between both forms is slow on the nmr time scale, and the presence of both conformers can be detected by inspection of the hydroxyl proton signals. Since the proportion of the minor conformer is small (<15%), the signals due to the protons attached to carbon in this form are obscured. Thus a unique situation exists; the low-temperature coupling constants reported for the trans isomer in Figure 1 are for the pure quasiequatorial (OH) form. Comparison of these values with those expected from the Karplus relationship shows excellent agreement for this molecule despite the presence of the electronegative hydroxyl groups.

Determination of the population of the two extreme conformers (C-1 hydroxyl quasiaxial *vs.* C-1 hydroxyl quasiequatorial) for the cis dihydrodiol proved to be a much more difficult problem. The coupling constants for the free diol



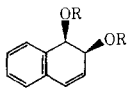
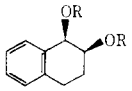
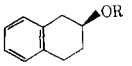
Isomer	Calculated Coupling Constants: C-1 Hydroxyl Quasiaxial	Calculated Coupling Constants: C-1 Hydroxyl Quasiequatorial	Observed Coupling Constants: Diol $CDCl_3$	Diol DMSO	Diol Acetone	Diacetate ($CDCl_3$)	Acetonide ($CDCl_3$)
Cis	$J_{1,2}$ 5.2	5.2	5.1	4.5	4.9	4.8	6.9
	$J_{2,3}$ 2.7	5.5	3.8	4.0	4.5	4.0	3.0
	$J_{2,4}$ −2.3	0.3	<0.5	<0.5	<0.5	1.3	−1.2
	Hydroxyls Quasiaxial	Hydroxyls Quasiequatorial					
Trans	$J_{1,2}$ 4.1	12.7	10.1	10.5	12.0	6.0	
	$J_{2,3}$ 5.5	2.7	2.5	2.2	1.6	4.0	
	$J_{2,4}$ 0.3	−2.3	−2.5	−2.5	2.3	1.0	

FIGURE 1: Proton magnetic resonance spectra of *cis*- (top) and *trans*- (bottom) 1,2-dihydroxy-1,2-dihydronaphthalene and the cis acetonide (middle) at 100 MHz. Only the carbinol and vinyl hydrogens which appear in the sequence H_4, H_3, H_1, and H_2 from left to right are shown. Calculated coupling constants (J) below are based on dihedral angles observed for extreme conformations of Dreiding stereomodels and the Karplus relationships presented by Becker (1969). Values of $J_{3,4}$ were all in the range of 9.75–10.0 Hz. The diols were exchanged with deuterium oxide prior to running spectra. Sign determinations were run for the observed coupling constants which are indicated as negative. Spectra in acetone were run at −70°. The values reported for the trans isomer in acetone at −70° are representative of a single conformer presumed to have the C-1 hydroxyl group quasiequatorial. While some dihydrodiols show conformational changes between DMSO and less polar solvents (Batterham and Young, 1969), the dihydrodiols of naphthalene studied here do not.

and the diacetate were not markedly different and were similar to those for the trans diacetate (Figure 1). Formation of an acetonide from the cis diol should result in a decrease of the dihedral angle between H_1 and H_2 with a resultant increase in J_{1-2}. A substantial increase from 5.1 Hz in the free diol to 6.9 Hz in the acetonide was observed. Seemingly, the acetonide exists with the C-1 oxygen substituent in a more axial average environment relative to the free diol since in the acetonide J_{2-4} could be detected and was established by a spin-tickling experiment as −1.2 Hz, while in the free diol this coupling was too small (<0.5 Hz) to be measured. The interconversion rate for the two conformers of the free diol was fast on the nmr time scale at −70°. Comparison of coupling constants in deuterated acetone at room temperature and at −70° showed J_{2-3} to increase slightly while J_{2-4} became more negative (sign assumed). Only the −70° values in acetone are reported (see table in Figure 1). Taken together these results suggest that both conformers of the free cis diol are present from room temperature to −70° and that the conformer with the C-1 hydroxyl quasiequatorial (as with the trans isomer) is favored by lowering temperature. Since the Karplus relationship was established to be applicable to this class of compounds with the trans isomer and since the bacterial dihydrodiol has coupling constants consistent with those predicted for cis relative stereochemistry, the bacterial diol must be *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. *P. putida* strain 119 produces only the cis stereoisomer. Careful examination of the mother liquors from crystallization of the metabolite produced in the large scale incubation provided no evidence by tlc that any trans isomer was formed.

The absolute stereochemistry and stereochemical purity of the (+)-cis dihydrodiol was established using the crude extract of metabolite obtained from incubation of *P. putida* strain 119 with naphthalene, in order to avoid enrichment in stereochemical purity by crystallization. Assignment was accomplished by reduction of the dihydrodiol to optically pure (−)-2(*S*)-hydroxy-1,2,3,4-tetrahydronaphthalene (Battail-Robert and Gagnaire, 1966) as shown in Table I and Scheme III. The somewhat lower optical purity previously reported (Jerina *et al.*, 1971a) was due to minor impurities in the final alcohol when this sequence was conducted on a very small scale. Since cis stereochemistry is present, the metabolite is optically pure (+)-*cis*-1(*R*),2(*S*)-dihydroxy-1,2-dihydronaphthalene. Interestingly, another strain of *P. putida* accumulates a dihydrodiol from toluene with related stereochemistry (Ziffer *et al.*, 1973; Kobal *et al.*, 1973).

Table I: Assignment of Metabolite as Optically Pure (+)-*cis*-1(*R*),2(*S*)-1,2-Dihydroxy-1,2-dihydronaphthalene.[a]

Compound	R_F, Solvent	$[\alpha]^{20}D$, deg (*c*, Solvent)	θ_{265}, Methanol
			
R = H	0.25, $CHCl_3$ (four developments)	+242 (1.00, $CHCl_3$) +220 (0.08, CH_3OH) +223 (1.04, DMSO)	+9583
R = acetonide	0.24, benzene	+147 (0.096, $CHCl_3$) +120 (0.080, CH_3OH)	+5417
R = acetate	0.70, $CHCl_3$	+94.4 (0.90, $CHCl_3$) +130 (0.08, CH_3OH)	+9509
			
R = H	(mp 129–130°)	−38 (0.87, $CHCl_3$)	
R = acetate	0.65, benzene–$CHCl_3$(1:1)	−167 (0.90, $CHCl_3$)	
			
R = acetate	0.55, benzene	−51.9 (0.99, $CHCl_3$)	
R = H	0.40, ethyl acetate–$CHCl_3$(1:4)	−67.2 (0.73, $CHCl_3$)[b]	

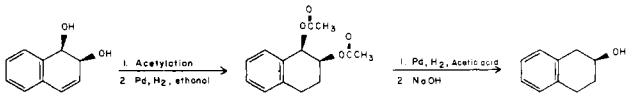
[a] Absolute stereochemistry is as shown. Proton magnetic resonance spectra are discussed in Figure 1. [b] A value of −67.1° has been reported (Battail-Robert and Gagnaire, 1966).

Table II: Stereochemistry of the Dihydrodiols Produced from Naphthalene by Different Bacterial Strains and by Rat Liver Microsomes.[a]

Incubation System	Rel. Sp Act. Cis	Trans
P. putida, Biotype B (ATCC 17484)	47	1
P. putida (wild type)	70	1
P. fluorescens (formerly, ATCC 17483)	110	1
Pseudomonas species (NCIB 9816)	461	1
Rat liver microsomes	1	40

[a] Experiment performed as described under Materials and Methods. Within the limit of error of the experiment, the bacteria produce only cis dihydrodiol and the rat liver microsomes produce only trans dihydrodiol.

SCHEME III: Assignment of Absolute Stereochemistry as (+)-*cis*-1(*R*),2(*S*)-Dihydroxy-1,2-dihydronaphthalene (The Enantiomers Involved Are Those Shown).



A method was sought to establish whether other available strains of bacteria, which metabolize naphthalene, do so by the initial formation of a cis dihydrodiol. As pointed out earlier, further metabolism of the diol by dehydrogenation prevents substantial accumulation of the diol in the incubation medium. However, radiochemical trapping experiments proved quite satisfactory. [^{14}C]Naphthalene together with cold carrier cis and trans dihydrodiols of naphthalene were incubated with extracts from cells of four different *Pseudomonas* species including the wild type isolated for this study, and the pools of carrier diol were reisolated and examined for radioactivity. Liver microsomes were run as a positive control for the production of the trans diol (Jerina *et al.*, 1970). Recovery of the carrier diols was satisfactory. For each of the bacteria, significant radioactivity could be detected only in the reisolated carrier cis diol (Table II).

The enzyme which produces the dihydrodiol from naphthalene in *P. putida* strain 119 was proved to be a dioxygenase (Hayaishi, 1964) by establishing that both atoms of oxygen in the diol arise from the same molecule of molecular oxygen when the *P. putida* strain 119 was incubated with naphthalene and a mixture of $^{16}O_2$ and $^{18}O_2$ gas (Table III). A similar experiment was achieved with *P. putida* (ATCC 17484), which does not accumulate the diol, by incubating [$^2H_8$]naphthalene in the presence of a large pool of unlabeled diol which prevents the dehydrogenase from oxidizing all of the newly formed diol containing both deuterium and labeled oxygen. Mass spectrometry of the recovered diol is easily interpreted since the molecular ion for newly formed deuterated diol appears at higher mass than any of the ions from the carrier.

The hydrocarbon substrate specificity of cell extracts prepared from naphthalene-grown cells of the *P. putida* was examined with ten aromatic hydrocarbons (Table IV). Phenanthrene was oxidized as rapidly as naphthalene, while benzene, substituted benzenes and naphthalenes, and anthracene showed 30–80% relative activity. No attempt was made to identify metabolites from these substrates. Only naphthalene supports significant growth of the organism.

In the presence of NADH, cell extracts oxidized naphthalene with the consumption of approximately 2 mol of oxygen per mol of naphthalene. Presumably, the first mol of oxygen is incorporated into the diol while the second mol is consumed during ring cleavage to *cis-o*-hydroxybenzalpyruvic acid (Scheme II) which was identified by its uv spectrum. When NADPH was used as the electron donor only 1

Table III: Origin of Oxygen Atoms in *cis*-Naphthalene-dihydrodiol.[a]

Organism	Oxygen Gas Composition		Isotope Composition of the Dihydrodiol	
	m/e	%	*m/e*	%
P. putida, strain 119	32	3.3	162	6.2
	34	15.1	164	15.2
	36	81.6	166	79.3
P. putida, strain 119	32	55.3	162	58.3
	34	6.8	164	7.0
	36	37.9	166	34.7
P. putida (ATCC 17484)	32	59.0	170[b]	60.3
	34	6.2	172	6.2
	36	34.8	174	33.5

[a] Intensities of the molecular ions of the dihydrodiols with different amounts of $^{18}O_2$ were obtained as described under Materials and Methods. [b] The [$^2H_8$]dihydrodiol formed in normal air and water has a nominal mass of 170. Peak heights are corrected for normal isotope abundances and for the fact that the naphthalene used was not completely deuterated (98%). The different samples of enriched oxygen gas used completely account for the ^{16}O–^{18}O present at the end of the incubations. The data are completely consistent with both oxygen atoms of the dihydrodiol arising from a single molecule of oxygen gas.

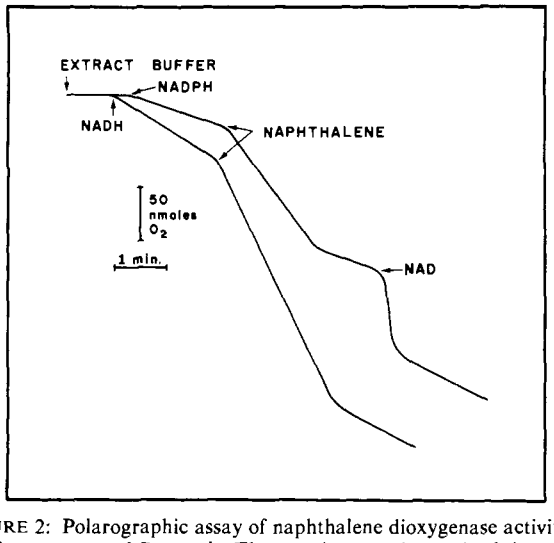


FIGURE 2: Polarographic assay of naphthalene dioxygenase activity in cell-free extracts of *P. putida*. The reaction vessel contained, in a final volume of 2.0 ml, phosphate buffer (pH 7.2) (100 μmol), NAD(P)H as indicated (0.35 μmol), naphthalene (0.1 μmol), and cell extract (7.0 mg of protein). At the end of naphthalene-stimulated NADPH oxidation, NAD^+ (0.5 μmol) was added to the reaction mixture.

mol of oxygen per mol of naphthalene was utilized. The addition of NAD^+ at the end of the reaction resulted in the further consumption of oxygen (Figure 2). When the reaction was examined spectrophotometrically, cell extracts catalyzed the oxidation of 1 mol of NADPH per mol of naphthalene. At the end of the reaction, the cuvet and its contents were made anaerobic and NAD^+ was added to the reaction mixture. There was an immediate production of NADH. The amount of NADH produced was equivalent to

Table IV: Relative Rates of Oxygen Consumption by Cell Extracts of *P. putida* in the Presence of NADH and a Variety of Aromatic Hydrocarbons.[a]

Substrate	Rel Act. (%)
Naphthalene	100
Benzene	29
Toluene	50
Ethylbenzene	47
Propylbenzene	26
1-Methylnaphthalene	61
2-Methylnaphthalene	80
Anthracene	41
Phenanthrene	100
Biphenyl	45

[a] The assay system in 2 ml final volume contained 50 mM phosphate buffer (pH 7.2), 0.5 μmol of NADH, 5.6 mg of extract protein, and 1.0 μmol of each substrate (in ethanol). The specific activities of the endogenous NADH oxidase and naphthalene oxygenase were 11 and 39, respectively. Specific activities are expressed as nanomoles of O_2 consumed/minute per milligram of protein.

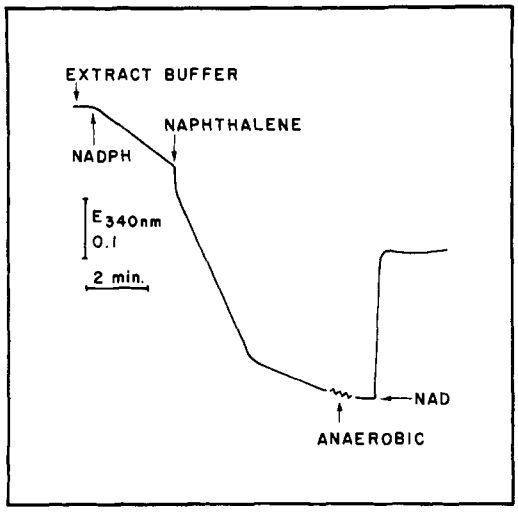


FIGURE 3: Spectrophotometric assay of naphthalene dioxygenase activity in cell-free extracts of *P. putida*. The reaction cuvet contained, in a final volume of 3.0 ml, phosphate buffer (pH 7.2) (150 μmol), NADPH (0.35 μmol), naphthalene (0.125 μmol), and cell extract (7.0 mg of protein). Upon completion of NADPH oxidation the cuvet and its contents were made anaerobic by alternately flushing and evacuating with nitrogen for 15 min. NAD^+ (0.5 μmol) was then added from the side arm of the cuvet at the point indicated.

the amount of naphthalene oxidized (Figure 3). The results indicated that the dioxygenase functions with either NADH or NADPH while the diol dehydrogenase is NAD^+ specific. In order for the second mol of oxygen to be consumed, the diol must be converted to 1,2-dihydroxynaphthalene in the presence of NAD^+. This interpretation was substantiated by the fact that the cis dihydrodiol could readily be isolated from incubations in which NADPH was the only added source of reducing equivalents.

Although a 1,2-dioxetane is an attractive intermediate (Scheme II, path a) to explain the stereochemistry and the oxygen incorporation in the cis dihydrodiol, no convincing evidence for such a species has been forthcoming either in

Table V: Oxidation of (+)- and (±)-*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene by the Purified NAD^+-Dependent Dihydrodiol Dehydrogenase.[a]

Substrate (μmol)	NADH Formed (μmol)
(+)-*cis*-Naphthalenedihydrodiol	
0.10	0.10
0.20	0.19
0.30	0.31
0.40	0.39
(±)-*cis*-Naphthalenedihydrodiol	
0.10	0.05
0.20	0.10
0.30	0.15
0.40	0.21

[a] Cuvets contained, in a final volume of 3.0 ml, phosphate buffer (pH 7.2, 280 μmol), NAD^+ (6.0 μmol), *cis*-naphthalenedihydrodiol as indicated, and dehydrogenase preparation (0.030 mg of protein). The reference cuvet contained all components except dihydrodiol. Cuvets were made anerobic as described in Figure 3. Reaction was initiated by combining substrate contained in a side arm of the cuvet with the other components. Rates and extent of reaction were monitored as described under Materials and Methods.

the present case (Jerina *et al.*, 1971a) or in the conversions of anthranilic acid (Kobayashi *et al.*, 1964), 2-fluorobenzoic acid (Milne *et al.*, 1968), or benzoic acid (Reiner and Hegeman, 1971) to catechol where such an intermediate has been suggested. Evidence for separate oxygenation and reduction steps has not been found in the present study. Furthermore, nonenzymatic cleavage of the strained naphthalene-1,2-dioxetane (Scheme II) would be expected to produce *o*-formylcinnamaldehyde. This compound has not been detected even though stable in the incubation medium. These results favor oxidation mechanisms involving metabolic intermediates that are bound to the enzyme at all times.

The NAD^+-dependent diol dehydrogenase present in *P. putida* has been purified to homogeneity (Patel and Gibson, 1974). The enzyme shows one-to-one stoichiometry between the amount of (+)-cis dihydrodiol consumed and NADH generated. With synthetic (±)-cis dihydrodiol at a saturating concentration, the rate was 90% of that observed for the (+)-cis isomer. This inhibition by the (−) isomer has not been studied in detail. However, at completion, the amount of NADH produced from the (±)-*cis*-naphthalenedihydrodiol was reduced by one-half, relative to that obtained from the (+) isomer (Table V). These results suggested that the enzyme was specific for the (+) isomer of *cis*-naphthalenedihydrodiol. A large scale incubation of the racemic dihydrodiol allowed isolation of (−)*cis*-1(*S*),2(*R*)-dihydroxy-1,2-dihydronaphthalene with $[\alpha]^{25}D$ −205° (*c* 0.05, methanol). While the rotation of the material was slightly less than expected for this optical antipode, the material had to be quite close to optical purity since no reaction could be detected when again subjected to the dehydrogenase.

A number of close structural analogs of (+)-cis-1(*R*),2(*S*)-dihydroxy-1,2-dihydronapthalene, the natural substrate, were examined to determine the specificity of the dehydrogenase. Detectable activity was not observed with (−)-*cis*-1(*R*),2(*S*)-dihydroxy-1,2,3,4-tetrahydronaphthalene, (±)-trans dihydrodiol, (±)-1- or 2-hydroxy-1,2-dihydronaphthalene, and (±)-1- or 2-hydroxy-1,2,3,4-tetrahydronaphthalene. The dehydrogenase is known to attack the cis dihydrodiols from a number of aromatic systems (Table IV; Patel and Gibson, 1974). Thus, the minimum structural requirement for the dehydrogenase is a cis dihydrodiol of an aromatic system. The total enantiomeric specificity observed with the cis dihydrodiol from naphthalene suggests, in addition, that (1) for fused polycyclic aromatic compounds, a *cis*-1(*R*),2(*S*)-dihydroxy-1,2,-dihydronaphthalene residue must be present, and that (2) for monsubstituted benzenes, the molecule must be a *cis*-1(*S*),2(*R*)-dihydroxy-1,2-dihydro-3-substituted benzene. Indications that the bacterial dioxygenases only form cis dihydrodiols with these stereochemical requirements have been previously noted (Ziffer *et al.*, 1973).

Deuterium-labeled (+)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalenes were employed in an attempt to distinguish between two extreme mechanistic possibilities for the NAD^+-dependent dihydrodiol dehydrogenase. The enzyme could function by a concerted elimination of hydrogen from C-1 and C-2 (Scheme IV, path a). Such a mechanism would show a primary kinetic isotope effect when deuterium was substituted at either C-1 or C-2 and should show an even larger kinetic isotope effect when both positions are substituted by deuterium. Alternatively, attack could occur by removal of hydrogen from the oxygen and carbon to form a ketone at either C-1 or C-2 which would rapidly enolize in a fast step (Scheme IV, path b). This mechanism would show a primary isotope effect when deuterium is substituted at either C-1 or C-2 depending upon which position is attacked. Substitution of deuterium at both positions

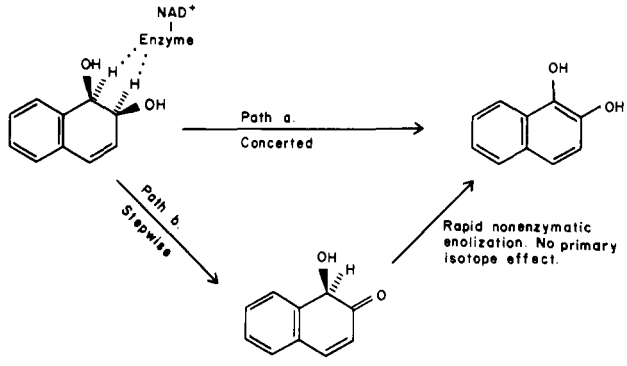
Table VI: Effect of Deuterium Substitution on the Enzymatic Oxidation of (+)-*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene.

Substrate	V_{max} (μmol min^{-1} $l.^{-1}$)[a]		Isotope Effect	Activation Energy (cal/mol)[b]	
	Obsd	Corrected[c]	k_H/k_D	Obsd	Corrected[c]
Unlabeled diol	9.33			4000	4000
Deuterated at C-1	8.89	8.50	1.09	3900	3800
Deuterated at C-2	7.54	4.97	1.88	4500	5200
Perdeuterated	5.25	4.97	1.88	5200	5200

[a] Apparent V_{max} values were obtained from Lineweaver-Burk plots of initial velocity *vs.* substrate concentration. Data were analyzed by a least-squares computer program. [b] Activation energies were calculated from the Arrhenius equation. [c] Observed values and values corrected to 100% deuteration at the indicated positions are presented.

would not show an increased isotope effect. Both pathways require carbon–hydrogen bond breaking to be involved in the rate-determining step in order to detect an isotope effect. The results obtained showed an isotope effect of $k_H/k_D \sim 1.9$ with an increase in activation energy of 1.15 kcal/mol for substitution of deuterium at C-2 and for substitution at both C-1 and C-2, while no significant change in rate was detected when deuterium was substituted at C-1 (Table VI). In addition, the diacetate of the (+)-cis dihydrodiol is not a substrate for the enzyme but is a potent competitive inhibitor of the free diol. These results favor the stepwise mechanism shown as path b in Scheme IV.

SCHEME IV: Possible Mechanism for *cis*-Naphthalenedihydrodiol Dehydrogenase (A Stepwise Attack for Path b Could Occur at C-1 As Well).



The bacterial dihydrodiol dehydrogenase is markedly different from a related enzyme in mammalian liver (Ayengar *et al.*, 1959) which also catalyzes the oxidation of dihydrodiols to catechols. The mammalian enzyme shows maximal activity with $NADP^+$ and will oxidize the cis dihydrodiol of benzene as well as both enantiomers of the trans dihydrodiol (Jerina *et al.*, 1970). The racemic trans dihydrodiols at the 1,2 position of naphthalene and the 9,10 position of phenanthrene were substrates. The mammalian enzyme appears to operate by the concerted mechanism (Scheme IV, path a) since an isotope effect was observed (F. Oesch, D. M. Jerina, and J. W. Daly, unpublished results) for oxidation of the trans dihydrodiol from naphthalene when deuterium was present at either C-1 or C-2. The isotope effect was cumulative when both positions were deuterated.

Acknowledgment

The authors express their appreciation to Professor W. C. Evans, The University College of North Wales, for a gift of *Pseudomonas* sp. NCIB 9816, to Dr. J. W. Daly of the National Institutes of Health for several helpful discussions, to Dr. H. Ziffer for circular dichroic measurements, to Dr. D. L. Rogerson of the National Institutes of Health for performing the large scale fermentation of naphthalene, and to Brigitte Gschwendt for technical assistance.

References

Augustine, R. L. (1965), Catalytic Hydrogenation, New York, N.Y., Marcel Dekker, p 135.

Ayengar, P. K., Hayaishi, O., Nakajima, M., and Tomida, I. (1959), *Biochim. Biophys. Acta 33,* 111.

Battail-Robert, D., and Gagnaire, D. (1966), *Bull. Soc. Chem. Fr. 33,* 208.

Batterham, T. J., and Young, I. G. (1969), *Tetrahedron Lett.,* 945.

Bauchop, T., and Elsden, S. R. (1960), *J. Gen. Microbiol. 23,* 457.

Becker, E. D. (1969), High Resolution NMR, New York, N.Y., Academic Press, p 104.

Booth, J., Boyland, E., and Turner, E. E. (1950), *J. Chem. Soc.,* 1188.

Bovey, F. A. (1969), Nuclear Magnetic Resonance Spectroscopy, New York, N.Y., Academic Press, p 152.

Boyland, E., and Levi, A. A. (1936), *Biochem. J. 30* 1225.

Canonica, L., Fiecchi, A., and Treccani, V. (1957), *Rend. Ist. Sci. Lett. Cl. Sci. Mat. Natur. 91,* 119.

Catterall, F. A., Murray, K., and Williams, P. A. (1971), *Biochim. Biophys, Acta 237,* 361.

Colla, C., Fiecchi, A., and Treccani, V. (1960), *Ann. Microbiol. Enzimol. 10,* 77.

Corner, E. D. S., and Young, L. (1954), *Biochem. J. 58,* 647.

Dagley, S. (1971), *Advan. Microbiol. Physiol. 6,* 1.

Daly, J. W., Jerina, D. M., and Witkop, B. (1972), *Experientia 28* 1129.

Evans, W. C. (1969), *in* Fermentation Advances, New York, N.Y., Academic Press, p 649.

Fieser, L. F., and Fieser, M. (1967), Reagents for Organic Synthesis, New York, N.Y., Wiley, p 268.

Gibson, D. T. (1971), *Crit. Rev. Microbiol. 1,* 199.

Griffiths, E., and Evans, W. C. (1965), *Biochem. J. 95,* 51.

Hayaishi, O. (1964), *Proc. 6th Int. Congr. Biochem. 33,* 31.

Jackman, L. M., and Sternhill, S. (1969), "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," 2nd ed, International Series of Monographs in Organic Chemistry, Vol. 5, Barton, D. H. R., and Doering, W., Ed., Elmsford, N.Y., Pergamon Press, pp 292–294.

Jeffrey, A. M., and Jerina, D. M. (1972), *J. Amer. Chem. Soc. 94,* 4048.

Jeffrey, A. M., Jerina, D. M., and Yeh, H. J. C. (1974), *J. Org. Chem. 39,* 1405.

Jerina, D. M. (1973), *Chem. Technol. 4,* 120.

Jerina, D. M., and Daly, J. W. (1974), *Science 185,* 573.

Jerina, D. M., Daly, J. W., Jeffrey, A. M., and Gibson, D. T. (1971a), *Arch. Biochem. Biophys. 142,* 394.

Jerina, D. M., Daly, J. W., and Witkop, B. (1971b), *Biochemistry 10,* 366.

Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S. (1970), *Biochemistry 9,* 147.

Karplus, M. (1963), *J. Amer. Chem. Soc. 85,* 2870.

Kobal, V. M., Gibson, D. T., Davis, R. E., and Garza, A. (1973), *J. Amer. Chem. Soc. 95,* 4420.

Kobayashi, S., Kuno, S., Itaka, N., and Hayaishi, O. (1964), *Biochem. Biophys. Res. Commun. 16,* 556.

Milne, G. W., Goldman, P., and Holtzman, J. (1968), *J. Biol. Chem. 243,* 5375.

Musliner, W. J., and Gates, J. W. (1966), *J. Amer. Chem. Soc. 88,* 4271.

Oesch, F., Jerina, D. M., Daly, J. W., Lu, A. Y. H., Kuntzman, R., and Conney, A. H. (1972), *Arch. Biochem. Biophys. 153,* 62.

Ornston, L. M. (1966), *J. Biol. Chem. 241,* 3800.

Patel, T. R., and Gibson, D. T. (1974), *J. Bacteriol. 119,* 879.

Reiner, A. M., and Hegeman, G. D. (1971), *Biochemistry 10,* 2530.

Stanier, R. Y., Palleroni, N. J., and Doudoroff, M. (1966), *J. Gen. Microbiol. 43,* 159.

Treccani, V. (1974), *in* Industrial Aspects of Biochemistry,

Spencer, B., Ed., Federation of European Biochemical Societies, p 533.
Walker, N., and Wiltshire, G. H. (1953), *J. Gen. Microbiol. 8*, 273.
Ziffer, H., Jerina, D. M., Gibson, D. T., and Kobal, V. M. (1973), *J. Amer. Chem. Soc. 95*, 4049.
Zobell, C. E. (1971), *Proc. Joint Conf. Prev. Contr. Oil Spills*, 441.

The Antibody–Enzyme Analogy. Characterization of Antibodies to Phosphopyridoxyltyrosine Derivatives†

Vic Raso‡ and B. D. Stollar*

ABSTRACT: Stable analogs of the crucial Schiff base intermediate of enzymatic and nonenzymatic pyridoxal phosphate catalysis have been used as haptens for induction of specific antibodies. *N*-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine and its conformationally distinct cyclized derivative resemble the Schiff base formed upon mixing tyrosine with pyridoxal phosphate. These compounds were covalently coupled to a protein carrier *via* the 3′-amino group so as to confer a prescribed orientation, with the coenzyme region farthest removed from the carrier. A third antigen, with the phosphopyridoxyl group alone as the hapten, was prepared by linkage of pyridoxal phosphate directly to free amino groups on the carrier protein. Antibodies elicited for each determinant were purified by means of appropriate affinity columns. Antibody heterogeneity was observed in that different species could be separated from a given serum by sequential elution from the affinity columns with 1 M sodium phosphate buffers of pH 7.6, 5.2, 2.6, and 1.5. In assays of quantitative precipitation, inhibition of precipitation, equilibrium dialysis, and fluorescence quenching, antibodies to the phosphopyridoxyltyrosine haptens showed specificity for the phosphorylated form of the coenzyme and binding activity for both the coenzyme and tyrosine portions of the hapten. Antibodies to the phosphopyridoxyl groups alone did not display a similar reactivity toward the tyrosine portion of the complex haptens. The cyclic and noncyclic conformations of the hapten were serologically distinct, as antibody to each reacted preferentially with the homologous form.

The relationship between enzyme and antibody proteins has long been of formal interest, since both types of proteins show similar kinds of specificity in binding small molecules–substrates, coenzymes, or haptens. While no catalytic activity has been associated with the binding of haptens by antibodies, it remained possible that a suitably designed antibody, with simultaneous specificity for a pair of substances which undergo slow spontaneous reactions, might demonstrate catalysis by virtue of binding the reactants in close proximity and suitable orientation on the protein surface. Such an antibody would be of value as a model in studying factors that contribute to the catalytic activity of enzymes.

The nature of the combining region of an antibody is prescribed to fit the chemical and steric features of the hapten determinant of an antigen. To elicit antibodies with enzyme-like binding specificity, we synthesized *P*-Pxy-Tyr(NH_2)[1] and its cyclized derivative, which are stable analogs of coenzyme–substrate Schiff base complexes, and we immunized rabbits with protein conjugates of these haptens.

Experiments described in this article demonstrate that the combining sites of the resulting antibodies did encompass both the coenzyme and the tyrosine portions of the hapten. Further experiments, described in the following article, showed that these analogs, which fit the antibody sites as haptens, also inhibit the enzymes tyrosine decarboxylase and tyrosine transaminase, that the condensation reaction between pyridoxal-*P* and tyrosine can proceed at the antibody combining site, that the resulting Schiff base is reversibly bound, but that the antibodies did not greatly accelerate Schiff base formation or reactions which proceed *via* this intermediate. Another antigen was also constructed by attaching the pyridoxal-*P* directly to a carrier protein; the antibodies induced by this conjugate did not have a similar specificity for tyrosine.

Materials and Methods

The synthesis and characterization of *P*-Pxy-Tyr(NH_2) and the cyclic *P*-Pxd<Tyr(NH_2) were previously described (Raso and Stollar, 1973). Deoxypyridoxine-*P* was synthesized and purified by the method of Peterson and Sober (1954). Pyridoxal-*P* and pyridoxamine-*P* were purchased from Sigma Chemical Company; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride from the Ott Chemical Company (Muskegon, Mich.); carboxymethylcellulose from H. Reeve Angel, Inc.; aminoethylcellulose from Nutritional Biochemical Company; Amberlite XE-64 from Rohm and Haas Company; complete Freund's adjuvant

† From the Molecular Basis of Biological Phenomena Program and the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111. *Received June 27, 1974.* This investigation was supported by Grants GB 29628 and GB 37937 from the National Science Foundation.

‡ Present address: Children's Cancer Research Foundation and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115.

[1] Abbreviations used are: *P*-Pxy-Tyr(NH_2), *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine; *P*-Pxd<Tyr(NH_2), cyclic *N*-(5-phosphopyridoxylidene)-3′-amino-L-tyrosine; *P*-Pxy-Tyr(NHAc), *N*-(5-phosphopyridoxyl)-3′-*N*-acetyl-3′-amino-L-tyrosine; P_i/NaCl, 0.15 M NaCl–0.005 M phosphate buffer (pH 8).