The Role of the Arene Oxide–Oxepin System in the Metabolism of Aromatic Substrates. IV. Stereochemical Considerations of Dihydrodiol Formation and Dehydrogenation[1]

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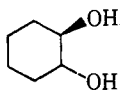
Abstract: Enzymatic hydration of cyclohexene oxide, styrene oxide, benzene oxide, 1,2-naphthalene oxide, and 9,10-phenanthrene oxide produces *trans* diols with varying degrees of enantiomeric homogeneity. With styrene oxide, attack by $H_2{}^{18}O$ occurs almost exclusively at the 2-position. Metabolism of benzene *in vivo* or hydration of benzene oxide *in vitro* forms an excess of the (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene enantiomer. The configuration was determined as 1*R*,2*R* by reduction to known (−)-*trans*-(1*R*,2*R*)-1,2-dihydroxycyclohexane. Comparison of circular dichroism curves allowed an assignment of 1*R*,2*R* stereochemistry for (−)-*trans*-1,2-dihydro-1,2-dihydroxy-4-chlorobenzene, an *in vivo* metabolite of chlorobenzene. *In vitro* hydration of the above mentioned oxides produces predominately the *R*,*R* enantiomer except in the case of 9,10-phenanthrene oxide where the *S*,*S* enantiomer predominates. Enzymatic dehydrogenation of the arene dihydrodiols to catechol is also stereoselective; the more readily dehydrogenated enantiomer is the minor isomer produced by the action of the hydrase on the arene oxide.

Arene oxides have been considered as possible intermediates in the oxidative metabolism and detoxification of aromatic compounds for several years,[2] but until recently there was no evidence for their formation in biological systems and no information about their metabolism. 1,2-Naphthalene oxide has now been identified as a microsomal metabolite of naphthalene.[1] In addition, both 1,2-naphthalene oxide[1] and benzene oxide[3] have been found to be substrates for a mammalian epoxide hydrase which converts them to (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene and (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene, respectively. Such *trans* dihydrodiols are also products of the *in vivo* metabolism of aromatic substrates such as benzene, naphthalene, and phenanthrene. Thus, the absolute stereochemistry of the dihydrodiols, formed either from arene oxides *in vitro* or from the arene itself *in vivo*, is significant with regard to the role of arene oxides in the oxidative metabolism of aromatic substrates. Since these *trans* dihydrodiols are substrates for a dehydrogenase which converts them to catechols,[4] it is also important to determine if dehydrogenation with this enzyme is stereoselective.

Discussion

The stereospecificity of microsomal "epoxide hydrase" has been investigated by examining the absolute stereochemistry of optically active *trans* diols produced from cyclohexene oxide, styrene oxide, benzene oxide, 1,2-naphthalene oxide, and 9,10-phenanthrene oxide. Under the conditions employed in these experiments, nonenzymatic opening of the oxides does not occur to any significant extent. The optical purity of the product, therefore, represents a measure of the stereoselectivity of the enzyme. The absolute stereochemistry of the arene dihydrodiols has been compared to the stereochemistry of the same diols formed by *in vivo* metabolism of the corresponding aromatic substrates.

Epoxide hydrase converts cyclohexene oxide to (−)-*trans*-1,2-dihydroxycyclohexane I with a high degree (~70%) of optical purity (Table I). The absolute stereochemistry of this compound has been determined[5] as 1*R*,2*R*. The literature contains several ref-



erences to the enzymatic hydration of epoxides[3,6–8] but this appears to be the first case in which the absolute stereochemistry of the product has been determined. There is evidence that microsomal oxidation of alkenes, such as 4-octene,[8] to the corresponding glycols proceeds *via* initial formation of epoxide and subsequent enzymatic hydration to the diol.

Enzymatic hydration of styrene oxide produces racemic phenylethane-1,2-diol[9] II with only a slight (<1%) excess of the (−)-1*R* enantiomer (Table I). Excluding the possibility of racemization, the optical purity of the diol II is a reflection of the low enzymatic stereoselectivity toward racemic styrene oxide since only ~5% of the observed conversion was obtained with denatured enzyme preparations. Two explanations for the low optical purity of the enzymatic product pertain; either the enzyme displays no positional preference and attack by water occurs in equal amounts at both the 1- and 2-positions of the oxide or the enzyme displays no significant preference for either enantiomeric form of the racemic substrate. The oxide was enzymatically hydrated in ^{18}O enriched water,[1] and over 90% of the ^{18}O was incorporated into the 2-hydroxyl group of the diol. This was established from the mass spectrum of diol II

(1) Part III; D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend, *J. Amer. Chem. Soc.*, 90, 6525 (1968).
(2) E. Boyland, *Biochem. Soc. Symp.*, 5, 40 (1950).
(3) D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend, *Arch. Biochem. Biophys.*, 128, 176 (1968).
(4) P. K. Ayengar, O. Hayaishi, M. Nakajima, and I. Tomida, *Biochim. Biophys. Acta*, 33, 111 (1959).
(5) Th. Posternak, D. Reymond, and H. Friedli, *Helv. Chim. Acta*, 38, 205 (1955).
(6) K. C. Leibman and E. Ortiz, *Mol. Pharmacol.*, 4, 20 (1968).
(7) K. C. Leibman and E. Ortiz, *Pharmacologist*, 10, 203 (1968).
(8) T. Watabe and E. W. Maynert, *ibid.*, 10, 203 (1968).
(9) See Table I, footnote *k*.

Table I. Conversion of Oxides to Diols with Rabbit Liver Microsomal Epoxide Hydrase[a]

Substrate (μmol)	% conversion to diol	Observed $[\alpha]^{25}D$, of diol, deg	$[\alpha]D$, of resolved diol, deg
Cyclohexene oxide (250)	2	−32.6[b]	−46.5[c]
Benzene oxide (100)	11	−250 ± 25[d]	(250)[e]
1,2-Naphthalene oxide (100)	30	−46.9 ± 4.7[f]	−159[g]
9,10-Phenanthrene oxide (50)	38	−87[h]	−138[i]
Styrene oxide (100)	18	−0.2[j]	62[k]

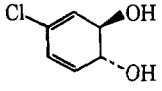
[a] The oxides were incubated with 10 ml of microsomal preparation and 2 ml of Tris buffer (pH 9.0, 0.5 *M*) for 10 min at 37°. Nonenzymatic hydration was not observed under these conditions (see Experimental Section). [b] The enzymatic *trans*-1,2-dihydroxycyclohexane was crystallized from ether. A weighted sample (2.2 mg in 10 ml of water) was used for the measurement; at 420 mμ, $[\alpha]^{25}_{420}$ −80°. The crude enzymatic product before crystallization had $[\alpha]^{25}D$ −21° (*c* 0.021, water); approximate optical purity, 70%. [c] N. A. B. Wilson and J. Reed, *J. Chem. Soc.*, 1269 (1935). [d] The value reported here (*c* 0.035, ethanol) represents a refinement of our earlier value.[3] [e] This is an estimate since the compound has not yet been prepared optically pure, but it is the highest $[\alpha]D$ which has been observed and does not increase on partial enzymatic dehydrogenation (see Table III). The *trans*-1,2-dihydroxycyclohexane obtained from the −250° dihydrodiol by reduction (20 mg of dihydrodiol in 10 ml of ethanol reduced with 5 mg of 5% palladium on barium sulfate and hydrogen gas) (M. Nakajima, I. Tomida, A. Hashizume, and S. Takei, *Chem. Ber.*, **89**, 2224 (1956)) had an $[\alpha]^{25}D$ −20.6° and $[\alpha]^{25}_{420}$ −49.7° (*c* 0.033, water) for material crystallized from ether. The crude reduction product had $[\alpha]^{25}D$ −23.6° (*c* ~0.200, water) assuming 100% reaction. Based on the known rotation of resolved *trans*-1,2-dihydroxycyclohexane, the (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene is roughly 44% optically pure. [f] An average value for two experiments (*c* 0.083, methanol). Approximately 32% optically pure. [g] J. Booth and E. Boyland, *Biochem. J.*, **44**, 361 (1949), E. D. S. Corner and L. Young, *ibid.*, **61**, 132 (1955). [h] This is a calculated value since insufficient metabolite was available for a direct measurement of $[\alpha]D$. The CD for the metabolite in methanol showed $[\theta]^{25}_{228}$ −12,660° compared with the reported value of −20,000° (footnote *i*) in which the solvent was not reported. If the solvent has little effect on the molar ellipticities, the metabolite has approximately 60% activity for resolved material. [i] R. Miura, S. Honmaru, and M. Nakazaki, *Tetrahedron Lett.*, 5271 (1968); J. Booth, E. Boyland, and E. E. Turner, *J. Chem. Soc.*, 2808 (1950). [j] This represents about 0.3% optical activity (*c* 0.130, methanol). [k] This is a calculated value based on data in I. Tömösközi, *Tetrahedron*, 1969 (1963), where (−)-mandelic acid [$[\alpha]D$ −104° (*c* 2.07, ethanol), optical purity 66%] was reduced to (−)-1-phenylethane-1,2-diol [$[\alpha]D$ −41.3° (*c* 3.043, ethanol)] which was analytically pure.

since the daughter ion $C_7H_8O^+$, resulting from benzylic cleavage accounts for less than 10% of the ^{18}O content observed in the molecular ion $C_8H_{11}O_2^+$. Thus, enzymatic hydration of styrene oxide shows topological specificity of hydration similar to that found for 1,2-naphthalene oxide in an earlier study,[1] and little enantiomeric specificity toward racemic styrene oxide.

Enzymatic hydration of benzene oxide forms (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene III. The absolute stereochemistry of III was determined by hydrogenation to (−)-*trans*-(1*R*,2*R*)-1,2-dihydroxycyclohexane I of known absolute stereochemistry.[5] It is difficult to estimate the stereoselectivity in benzene oxide opening, since *trans*-1,2-dihydro-1,2-dihydroxybenzene has not been resolved. However, based on the optical activity associated with the reduction product, the dihydrodiol was at least 50% optically pure. Thus the epoxide hydrase converts both cyclohexene oxide and benzene oxide to *trans* diols with 1*R*,2*R* configurations.

Trace amounts of *trans*-1,2-dihydro-1,2-dihydroxybenzene have been observed as an *in vivo* metabolite (rabbit) of benzene.[10] The optical activity of the diol was not reported. Repetition of this work led to the isolation of a small amount of diol[11] whose CD curve was virtually identical with that of the diol obtained *in vitro* from benzene oxide.

The absolute stereochemistry of (−)-*trans*-1,2-dihydro-1,2-dihydroxy-4-chlorobenzene IV, obtained as an *in vivo* metabolite of chlorobenzene in rabbits,[12,13] has been assigned by an examination of its circular dichroism (CD) curve. The sign of the Cotton effect associated with the longest wavelength transition of



conjugated dienes has been shown to be related to the skew sense of the diene.[14] The absolute stereochemistry of such dienes can be assigned directly provided either the skew sense of the diene is unequivocal or, if the skew sense is equivocal, due to a conformational equilibrium, the concentration and rotational strength of each rotational conformer is known. Diol IV is of this latter type; the skew sense of the diene is dependent upon the hydroxyl groups being quasi-axial or quasi-equatorial. Consequently, the sign of the CD curve cannot be used directly to assign the configuration of the asymmetric centers without some knowledge of the conformational equilibrium. Unfortunately, little information is available on the relative stabilities of quasi-axial *vs.* quasi-equatorial substituents in substituted cyclohexadienes. The energy differences between axial and equatorial hydroxyl groups decrease in going from cyclohexane to cyclohexene.[15] Presumably the introduction of a second double bond would further decrease this difference. However, since the absolute stereochemistry of III is known, the sign of its CD curve can be used to determine, in effect, the preferred conformation or that conformation associated with the greatest rotational strength. Introduction of the chloro substituent on the diene is not likely to affect significantly the preferred conformation or the rotational strength associated with these conformations. The observed negative CD curve for III (Figure 1) requires the conformation associated with quasi-axial hydroxyl groups to be either the preferred conformation or that associated with the greatest rotational strength. The results of studies on related systems support this conclusion. For example, an investigation of the temperature-de-

(10) T. Sato, T. Fukuyama, T. Suzuki, and H. Yoshikawa, *J. Biochem.*, **53**, 23 (1963).

(11) Other workers [I. Tomida and M. Nakajima, *Hoppe-Seyler's Z. Physiol. Chem.*, **318** 171 (1960)] using a different isolation procedure were unable to detect this metabolite of benzene.

(12) D. M. Jerina, J. W. Daly, and B. Witkop, *J. Amer. Chem. Soc.*, **89**, 5488 (1967).

(13) J. N. Smith, B. Spencer, and R. T. Williams, *Biochem. J.*, **47**, 284 (1950).

(14) U. Weiss, H. Ziffer, and E. Charney, *Tetrahedron*, 3105 (1968).

(15) M. Hanack, "Conformation Theory," Academic Press, New York, N. Y., 1965, p 146.

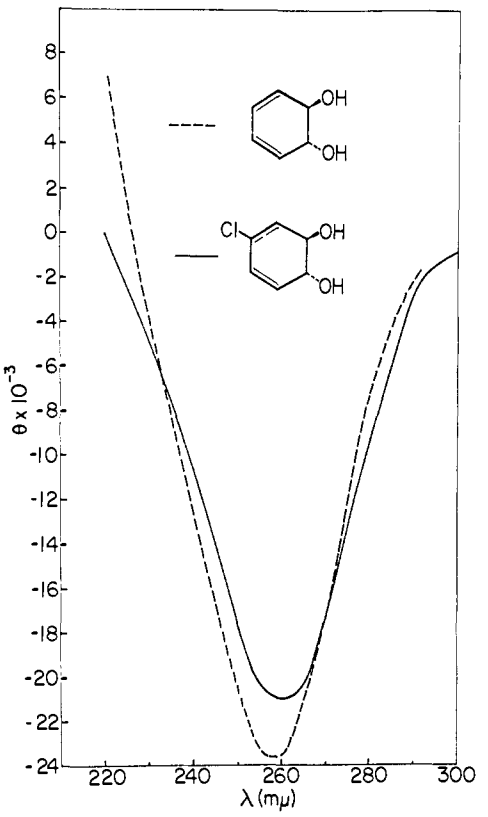
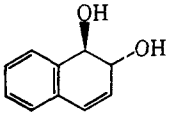


Figure 1. Circular dichroism curve for III, (−)-(1*R*,2*R*)-*trans*-1,2-dihydro-1,2-dihydroxybenzene (*c* 0.0036, ethanol) and IV, (−)-(1*R*,2*R*)-*trans*-1,2-dihydro-1,2-dihydroxy-4-chlorobenzene (*c* 0.0204, water). The optical purity of these samples is unknown.

pendent rotation of (−)-α-phellandrene[16] demonstrated that the conformation of the molecule with a quasi-axial isopropyl group determined the sign of the CD curve. Miura, *et al.*,[17] concluded from a study of the ORD (and CD) curves of (−)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene that the preferred conformation or the conformer associated with the greater rotational strength, was the one in which the hydroxyl groups were quasi-axial. In examining the solvent dependence of the nmr spectrum of *trans*-1-hydroxy-2-hydroxymethyl-3-cyano-4-amino-$\Delta^{3,5}$-cyclohexadiene, Johnson and Heischen[18] found that the coupling constant for the hydrogens on carbons 1 and 2 were best explained by assuming a conformational equilibrium in which the quasi-axial hydroxyl and the hydroxymethyl groups made a significant contribution. The essentially identical magnitudes of the CD curves of III and IV (Figure 1) strongly support the assumption that both compounds have the same preferred conformation or conformational equilibrium. Since the CD curves have the same sign, the absolute stereochemistry at the carbinol positions of both compounds must be identical.

Thus *in vivo* metabolism of benzene and chlorobenzene leads to the formation of *trans*-dihydrodiols with the same configuration *R*,*R* as the diol obtained from benzene oxide on enzymatic hydration *in vitro*. Synthetic 4-chlorobenzene 1,2-oxide is as yet not available for an investigation of its enzymatic hydration. It would appear likely that *in vivo* metabolism of monocyclic arenes to *trans* dihydrodiols proceeds *via* the corresponding arene oxides but it has not yet been possible to demonstrate *in vitro* formation of arene oxides or dihydrodiols from monocyclic aromatic substrates.[3] The *in vitro* conversion of the bicyclic substrate, naphthalene, to both 1,2-naphthalene oxide and the corresponding dihydrodiol has, however, been reported.[1]

The absolute stereochemistry of the diol formed *in vitro* from naphthalene or from 1,2-naphthalene oxide was now investigated. As shown in Table II, (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene V is formed by microsomes with all the species studied using either



naphthalene or 1,2-naphthalene oxide as the substrate. The diols formed *in vitro* from either substrate have similar (~30–50%) optical purity (Table III). Earlier investigations[1] indicated that naphthalene oxide is a requisite intermediate in the *in vitro* conversion of naphthalene to *trans*-1,2-dihydro-1,2-dihydroxynaphthalene.

Table II. Enzymatic Formation of *trans*-1,2-Dihydro-1,2-dihydroxynaphthalene from Naphthalene and from 1,2-Naphthalene Oxide with Liver Microsomes[a]

Source of liver microsomes	$[\alpha]^{25}D$, (MeOH) *trans*-1,2-dihydro-1,2-dihydroxynaphthalene	
	From naphthalene[b]	From 1,2-naphthalene oxide[c]
Rat	−66.0	−51.8
Rabbit	−53.5 ± 4.6	−46.9 ± 4.7
Guinea pig	−96.4	−88.0
Mouse	−75.4 ± 7.0	−68.8

[a] *In vivo* dihydrodiol formation from naphthalene with the species studied here has led to varying stereochemical results. Rat and rabbit excrete mainly racemic diol in the urine along with slight excess of the − form for the rat and + form for the rabbit [J. Booth and E. Boyland, *Biochem. J.*, **44**, 361 (1949)]. Male guinea pigs excrete mainly the − form [E. D. S. Corner and L. Young, *ibid.*, **58**, 647 (1954)]. Only the mouse has been studied *in vitro* using microsomes [J. R. Holtzman, J. R. Gillette, and G. W. A. Milne, *J. Biol. Chem.*, **242**, 4368 (1967), and *J. Amer. Chem. Soc.*, **89**, 6341 (1967)]. In that study almost optically pure + form was observed; $[\alpha]^{25}D$ +159° (*c* 1.00, ethanol) and $[\alpha]^{25}D$ +412° (*c* 0.66, chloroform). Under our conditions and the conditions of the earlier study using naphthalene or naphthalene oxide, we observed excesses of the − form on *several* trials with mouse microsomes. [b] Naphthalene (10 mg) was incubated with 10 ml of microsomal preparation, 3 ml of pH 8 Tris buffer, NADP, ATP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase for 30 min (see Experimental Section). [c] 1,2-Naphthalene oxide (8 mg) was incubated under nitrogen with 10 ml of microsomal preparation and 2 ml of pH 9 Tris buffer for 10 min (see Experimental Section).

Table III. Partial Resolution of (±)-*trans*-1,2-Dihydro-1,2-dihydroxybenzene by Enzymatic Dehydrogenation

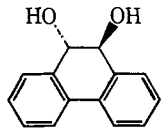
% conversion	$\theta_{257.5}$[a]
15	−2,732
22	−7,676
32	−12,130

[a] Average value of $\theta_{257.5}$ for the dihydrodiol produced by the action of hydrase on the epoxide is −20,700 ± 1600°. Partial enzymatic dehydrogenation showed no increase in optical activity.

(16) H. Ziffer, E. Charney, and U. Weiss, *J. Amer. Chem. Soc.*, **84**, 2961 (1962).
(17) See Table I, footnote *i*.
(18) F. Johnson and J. P. Heischen, *J. Org. Chem.*, **29**, 3252 (1964).

These stereochemical studies are in accord with this conclusion. The sign of rotation had been related to the absolute stereochemistry for the naphthalene diol V, and the − enantiomer was shown to have the 1*R*,2*R* configuration.[17] Determination of the CD curve of diol V (Figure 2) allowed a measurement of the optical activity in the samples reported here for comparison with earlier data on this diol which had all been reported as [α]D. Enzymatic hydration was found to be less stereoselective with naphthalene oxide than with cyclohexene oxide or benzene oxide. However, the *R*,*R* configuration (~30–50% optical purity) about the diol still obtains. The low enantiomeric preference in the hydration of naphthalene oxide may account for the observation that *in vivo* formation of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene from naphthalene most often leads to racemic material with, depending on species, a slight excess of one or the other of the enantiomers (see Table II) in the urine.

When 9,10-phenanthrene oxide was investigated with microsomal preparations, hydration to diol was, as in the case of cyclohexene oxide, benzene oxide, and naphthalene oxide, completely enzymatic. Considerable isomerization to 9-hydroxyphenanthrene was also noted. An earlier study[19] using much longer incubations reported only nonenzymatic hydration and rearrangement to the phenol. The enzymatically formed diol was identical with (−)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene. As seen from Table I, this material was strongly levorotatory, having 60–70% of the optical activity associated with resolved material VI. The sign of rotation had been related to its absolute stereochemistry,[17] and the configuration of the major steroisomer produced by enzymatic hydration is thus 9S, 10S. This contrasts with the *in vitro* hydration of the oxides from cyclohexene, benzene, and naphthalene



which produced mainly the *R*,*R*-*trans*-diols (Table I). As with naphthalene, the *in vivo* results with phenanthrene are difficult to interpret. Rats and rabbits generally excrete mostly racemic material along with a slight excess of the (−)-form in the urine.[20,21]

Such arene dihydrodiols are further metabolized by a soluble liver enzyme[4] which dehydrogenates them to catechols. This dehydrogenase has now been found to be quite stereoselective. Racemic *trans*-1,2-dihydro-1,2-dihydroxybenzene was subjected to varying amounts of dehydrogenation. Unreacted starting material was then isolated from the mixture and the molar ellipticity of each sample measured. As the per cent conversion increased, so did the proportion of the (−)-dihydrodiol III in the recovered starting material (Table III). That is, the enantiomer which is selectivity produced during benzene oxide hydration is *not* the enantiomer which is selectively dehydrogenated. The + enantiomer is dehydrogenated at a rate severalfold greater than − enantiomer III. The same specificity was observed during dehydrogenation of racemic *trans*-1,2-dihydro-1,2-

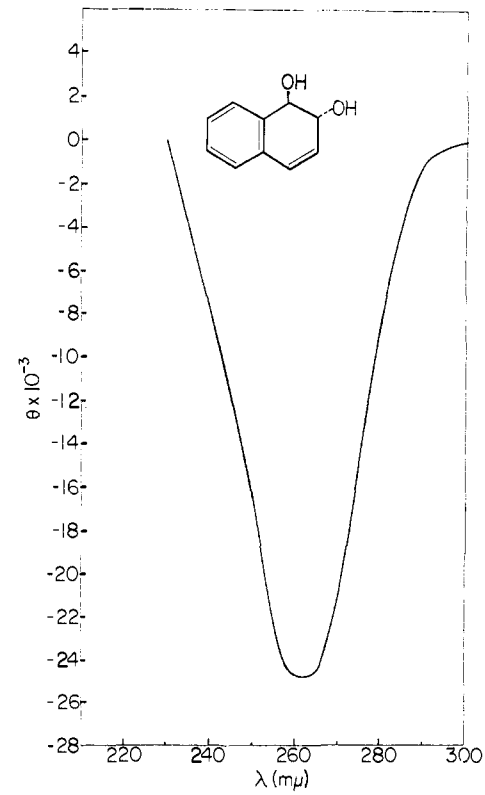


Figure 2. Circular dichroism curve for V, (−)-*trans*(1*R*,2*R*)-1,2-dihydro-1,2-dihydroxynaphthalene (*c* 0.803, methanol). The optical purity of this sample is 25%.

dihydroxynaphthalene and *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene. In each case, an excess of the − enantiomer was present after partial dehydrogenation. The phenanthrene dihydrodiol was an extremely poor substrate for the dehydrogenase, while both enantiomers of the naphthalene dihydrodiol were rapidly dehydrogenated. Thus, the dehydrogenase is more selective for the minor isomer produced by the epoxide hydrase with all the substrates studied; *i.e.*, the preference for a certain absolute stereochemistry changes for both enzymes when the phenanthrene substrates are employed. This may reflect a change in the mode of binding on both the hydrase and the dehydrogenase with the phenanthrene substrates.

There are several tentative conclusions which may be drawn from these studies. Comparison of the dihydrodiol from benzene and chlorobenzene *in vivo* showed the major stereoisomer *R*,*R* to be identical with that produced *in vitro* from benzene oxide. This result is compatible with the suggestion that arene oxides are *in vivo* intermediates in benzene and chlorobenzene metabolism. Similar conclusions might be drawn with regard to naphthalene and phenanthrene *in vivo vs.* 1,2-naphthalene oxide and 9,10-phenanthrene oxide *in vitro*, were it not for the fact that the major stereoisomers of the dihydrodiols produced *in vivo* from the corresponding arenes vary with species. This variation could be the result of further metabolism, but it has been shown that administration of −, +, or ± dihydrodiol of naphthalene to male hooded rats results in excretion of unconjugated diol having unchanged optical properties.[22] Similarly, administration of racemic *trans*-9,10-dihydro-

(19) E. Boyland and P. Sims, *Biochem. J.*, **95**, 788 (1965).
(20) E. Boyland and G. Wolf, *ibid.*, **47**, 64 (1950).
(21) E. Boyland and P. Sims, *ibid.*, **84**, 571 (1962).
(22) E. D. S. Corner and L. Young, *ibid.*, **61**, 132 (1955).

Table IV. Enzymatic Dehydrogenation of Dihydrodiols[a]

Substrate (5 μmol)	Time, min	% conversion to catechol	Sign of cotton effect[b]
(±)-*trans*-1,2-Dihydro-1,2-dihydroxybenzene	20	45	Negative
(−)-*trans*-1,2-Dihydro-1,2-dihydroxybenzene[c]	20	8	Negative
cis-1,2-Dihydro-1,2-dihydroxybenzene	20	13	
(±)-*trans*-1,2-Dihydro-1,2-dihydroxynaphthalene	10	72	Negative
(±)-*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene[d]	60	6	Negative

[a] The dihydrodiol was incubated with, for varying times, 4 ml of rabbit liver soluble fraction, 1 ml of pH 8 Tris buffer, and NADP, and the disappearance of dihydrodiol was measured (see Experimental Section). [b] In general, insufficient material was obtained to make reliable $[\alpha]_D$ measurements. [c] This material was obtained by hydration of benzene oxide and had $[\alpha]_D$ −250°. [d] Since this compound dehydrogenates so slowly, 10 μmol of substrate was used.

9,10-dihydroxyphenanthrene to rats resulted in urinary excretion of the unconjugated racemic diol.[23] A slight excess of the glucuronide of the − diol was, however, noted in this latter experiment.

The data further suggest that enzymatic formation of arene oxides is not a particularly stereoselective process. For example, consider the metabolism of naphthalene which produces mainly racemic diol. Formation of this racemic *trans* diol has been shown to occur by addition of water to only the 2-position of the oxide.[1] Since geometry about C-1 must be maintained during this process, it is necessary that enzymatic oxidation of naphthalene produces both stereoisomeric forms of 1,2-naphthalene oxide which are then hydrated to produce the observed racemic diol. Whether both stereoisomeric forms of 1,2-naphthalene oxide are produced enzymatically or whether optically active oxide can invert *via* the oxepin form is currently under study.

The fact that the epoxide hydrase does not produce the stereoisomer of the dihydrodiols which is most easily converted to a catechol by the soluble dehydrogenase was entirely unexpected. This correlation of stereospecifications between the two enzymes holds true with all the substrates studied; even with the phenanthrene diol in which the absolute stereochemistry of the major isomer formed by epoxide hydrase changed from *R,R* to *S,S*. The significance of the interrelationship between these two enzymes to the overall metabolism of aromatic substrates is as yet unclear.

Experimental Section

Optically inactive benzene oxide,[3] 1,2-naphthalene oxide,[24] 9,10-phenanthrene oxide,[25] *cis*-[26] and *trans*-1,2-dihydro-1,2-dihydroxybenzene,[27] *trans*-1,2-dihydro-1,2-dihydroxynaphthalene,[28] and *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene[28] were prepared as previously described. *trans*-1,2-Dihydroxycyclohexane was resolved[29] *via* the menthoxyacetate ester.

In Vivo **Metabolites.** The administration of chlorobenzene[12,13] to rabbits yielded (−)-*trans*-1,2-dihydro-1,2-dihydroxy-4-chlorobenzene as a urinary metabolite.

Intraperitoneal injection of benzene (2 ml of a 50% solution in cottonseed oil/animal) into six rabbits (New Zealand white females) led to the isolation of (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene following the activated charcoal adsorption procedure of Sato, *et al.*[10] The final purification of this compound was by tlc as described below. Spectroscopic estimation of the amount of the metabolite (<0.1 mg) was difficult due to the presence of a light-absorbing contaminant. The shape and sign of the CD (circular dichroism) curve was, however, identical with that observed for diol produced by enzymatic hydration of benzene oxide (Figure 1).

Preparation of Liver Subcellular Fractions. Liver (1 part) homogenates with isotonic KCl (3 parts) were centrifuged at 15,000 g for 30 min to remove nuclei and mitochondria and at 100,000 for 1 hr to obtain a soluble fraction and a microsomal pellet. The pellet was suspended in 5 vol of isotonic KCl, to yield the microsomal preparation.

Enzymatic Hydration of Epoxides. The epoxide (10–25 mg) in 0.2 ml of ethanol was added to a mixture of 10 ml of microsomal preparation and 2 ml of Tris buffer (pH 9.0, 0.5 *M*) containing 2 mg of Tween 80. The mixture was incubated for 10 min at 37° under nitrogen and then extracted twice with equal volumes of ethyl acetate. In the case of cyclohexene oxide, the protein was precipitated by addition of methanol, removed by centrifugation, and the supernatant was taken to dryness partially *in vacuo* and then by lyophilization. The residue was extracted with ethyl acetate. Ethyl acetate extracts were dried (Na_2SO_4), concentrated, and chromatographed on silica gel GF thin layer chromatoplates (Analtech, Inc.) with benzene–chloroform–ethyl acetate (1:1:1). The metabolites were detected under ultraviolet light or in the case of 1,2-dihydroxycyclohexane with 8 *N* chromium trioxide in sulfuric acid. The products were extracted from silica gel scrapings with ethyl acetate and purified by crystallization or rechromatography. In order to obtain sufficient *trans*-1,2-dihydro-1,2-dihydroxybenzene, *trans*-1,2-dihydroxycyclohexane, and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, incubations with these substrates were repeated on a fivefold larger scale. The R_f values on tlc were: *trans*-1,2-dihydro-1,2-dihydroxybenzene (0.20); *trans*-1,2-dihydro-1,2-dihydroxynaphthalene (0.25); *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (0.40); 1-phenylethane-1,2-diol (0.26); and *trans*-1,2-dihydroxycyclohexane (0.15). The styrene oxide, benzene oxide, 9,10-phenanthrene oxide, and 1,2-naphthalene oxide were excellent substrates for the epoxide hydrase while the cyclohexane oxide was a rather poor substrate[3] (Table I). Control incubations with boiled enzyme indicated a small amount (~5% of enzymatic conversion) of nonenzymatic hydration with styrene oxide but not with any of the other substrates. The diols isolated from enzymatic hydration displayed, in all cases, a negative Cotton effect (Table I).

Enzymatic Dehydrogenation of Dihydrodiols. The arene dihydrodiols were dehydrogenated in the following manner: substrate (5 μmol in 0.2 ml of ethanol) was added to a mixture of 4 ml of the soluble fraction prepared from rabbit liver, 5 mg of NADP, and 1 ml of Tris buffer (pH 8.0, 0.5 *M*). The mixture was incubated for 10–60 min at 37° and the unreacted arene dihydrodiol was recovered by extraction and tlc as described above. A zero time incubation served as control. Recoveries of unreacted arene dihydrodiols and rotations are given in Tables III and IV.

Microsomal Oxidations. Microsomal hydroxylation of naphthalene was carried out as follows: naphthalene (10 mg in 0.1 ml of ethanol) was added to 10 ml of microsomal preparation, 3 ml of Tris buffer (pH 8.0, 0.5 *M*) containing 2 mg of Tween 80, 15 μmol of NADP, 5 μmol of ATP, 50 μmol of glucose-6-phosphate, and 10 units of glucose-6-phosphate dehydrogenase, and incubated at 37° for 30 min. The dihydrodiol was extracted into ethyl acetate and isolated as described above. The results using microsomes from several species are shown in Table II.

Optical Activity Measurements. Rotations were measured with a Perkin-Elmer 141 polarimeter using a 10-cm cell holding 1 ml of solvent. Circular dichroism (CD) curves were obtained on a Cary 60. Details for each measurement are presented with the tables and figures. It is important to realize that many of the values have

(23) E. Boyland and P. Sims, *Biochem. J.*, **84**, 583 (1962).
(24) E. Vogel and F. G. Klärner, *Angew. Chem. Intern. Ed. Engl.*, **7**, 374 (1968).
(25) M. G. Newman and S. Blum, *J. Amer. Chem. Soc.*, **86**, 5598 (1964).
(26) M. Nakajima, J. Tomida, and S. Takei, *Chem. Ber.*, **92**, 163 (1959).
(27) See Table I, footnote *e*.
(28) J. Booth, E. Boyland, and E. E. Turner, *J. Chem. Soc.*, 1188 (1950).
(29) See Table I, footnote *c*.

been obtained on very small amounts of material, and it is the sign of the rotation and not its exact magnitude that is significant.

Acknowledgment. The authors express their gratitude to Professor M. Nakajima of Kyoto University for samples of *cis*- and *trans*-1,2-dihydro-1,2-dihydroxybenzene, to Dr. K. Kirk of this laboratory for a sample of 9,10-phenanthrene oxide, and Dr. J. Holtzman, NIH, for a sample of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene. In addition we thank Dr. Bernhard Witkop for his continued interest and encouragement.

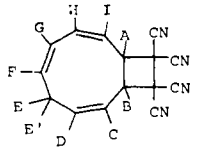
Communications to the Editor

Cycloaddition Reactions of Bicyclo[6.1.0]nona-2,4,6-triene

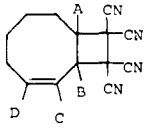
Sir:

There has been considerable interest recently in establishing the mechanism of the thermal reorganization of *cis*-bicyclo[6.1.0]nona-2,4,6-triene (**1**) based on orbital symmetry considerations.[1] The cycloaddition reactions of **1**, however, have received little attention.[2] Since orbital symmetry is inherently related to electrocyclic, sigmatropic, and cycloaddition reactions,[3] we felt it important to obtain information regarding the mode of cycloaddition of **1** with several dienophiles.

Tetracyanoethylene (TCNE) reacts with **1** (refluxing tetrahydrofuran, 2 hr)[4] in a remarkable way to produce the adduct, tentatively assigned structure **2**. Even a cursory examination of the nmr spectrum of the adduct, mp 140.5–141.0° (56%),[4] indicates the presence of six



2

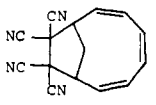


3

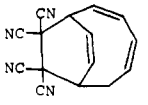
olefinic protons and four other protons. The structure of the adduct corresponds *formally* to an adduct produced by the cycloaddition of TCNE to a cyclononatetraene.[5] The uv spectrum (in ethanol) of the adduct reveals a shoulder at 225 mμ (log ε 3.86) with end absorption, which suggests the presence of a conjugated diene in an eight- or nine-membered ring.[6] The infrared spectrum appears to lack *trans* double bond absorption.

The nmr spectrum (60 MHz; $CDCl_3$) of **2** shows six olefinic protons at τ 3.59 (1 H, d, $J \sim 12.3$ Hz), 3.9–4.3 (4 H, m), and 4.36 (1 H, dd, $J \sim 11.1, 2.6$ Hz) and four other protons at τ 6.09 (1 H), 6.36 (1 H), and 7.3–7.6 (2 H). On the assumption that **2** is correctly assigned, the resonances at τ 6.36 and 6.09 can be assigned to H_A and H_B, respectively, and on more careful examination, they appear to constitute an AB pattern ($|J_{AB}| \sim 10.7$ Hz). The double doublet at τ 4.36 is assigned to H_C, because irradiation of H_B, but not H_A, causes its smaller coupling ($|J_{BC}| \sim 2.6$ Hz) to disappear. The irradiation of the low-field doublet (τ 3.59) assigned to H_I produces only slight sharpening of H_A, but no other changes in the high-field resonances. Conversely, irradiation of H_A produces only slight sharpening of H_I. Careful irradiation of the highest field resonances (H_E, $H_{E'}$) produces considerable sharpening, but no interpretable changes, in the region τ 3.9–4.3, which presumably contains the resonances due to H_D, H_F, H_G, and H_H. Furthermore, H_E does not appear to be coupled to H_A or H_B. The ABC pattern in the spectrum of **2** is similar to that of **3**, which was kindly communicated to us by Dr. J. J. Bloomfield.[7] The coupling constants for **3** observed by Bloomfield were $|J_{AB}| \sim 9.5$ Hz, $|J_{CD}| \sim 11$ Hz, and $|J_{BC}| \sim 4$ Hz, and the protons H_A, H_B, and H_C appeared (in dimethyl sulfoxide) at τ 6.2, 5.7, and 4.4, respectively.

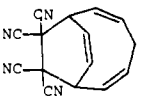
Besides **2**, other structures, **4a–d**, were also considered. The symmetrical structures **4a** and **4c** are ruled out by



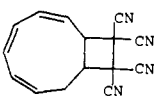
4a



4b



4c



4d

the lack of symmetry in the observed nmr spectrum. **4a** and **4d** appear inconsistent with the uv spectrum. **4b** and **4d** appear to be ruled out, because the observed methylene protons appeared not to be coupled (nmr) to either of the methine protons, whereas such a coupling might be expected, especially in the case of **4b**.

(1) S. W. Staley and T. J. Henry, *J. Amer. Chem. Soc.*, **91**, 1239 (1969), and P. Radlick and W. Fenical, *ibid.*, **91**, 1560 (1969), for leading references. We thank Professor Staley for providing us with the details of his excellent method for synthesizing **1**.

(2) Several adducts of bicyclo[6.1.0]nona-2,4,6-trienes have been reported. See (a) T. J. Katz and P. J. Garratt, *ibid.*, **86**, 5194 (1964); (b) E. A. LaLancette and R. E. Benson, *ibid.*, **87**, 1941 (1965); (c) T. S. Cantrell and H. Schechter, *ibid.*, **89**, 5868 (1967); (d) A. C. Cope, P. T. Moore, and W. R. Moore, *ibid.*, **80**, 5505 (1958); (e) D. Schoenleber, *Chem. Ber.*, **102**, 1789 (1969).

(3) (a) R. B. Woodward and R. Hoffmann, *J. Amer. Chem. Soc.*, **87**, 395, 2511 (1965); (b) R. Hoffmann and R. B. Woodward, *ibid.*, **87**, 2046, 4388, 4389, (1965); (c) H. C. Longuet-Higgins and E. W. Abrahamson, *ibid.*, **87**, 2045 (1965); (d) R. Hoffmann and R. B. Woodward, *Accounts Chem. Res.*, **1**, 17 (1968); (e) R. B. Woodward, Special Publication No. 21, The Chemical Society, London, 1967, pp 217–249.

(4) We thank Professor Katz for some comparison data.[2a]

(5) The *all-cis*-cyclonona-1,3,5,7-tetraene has been prepared [P. C. Radlick and G. Alford, *J. Amer. Chem. Soc.*, **91**, 6529 (1969)]. See also, A. G. Anastassiou, *Chem. Eng. News*, **47** (39), 48 (1969); V. G. Boche, H. Böhme, and D. Martens, *Angew. Chem.*, **81**, 565 (1969); and S. Masamune, P. M. Baker, and K. Hojo, *Chem. Commun.*, 1203 (1969).

(6) Cyclonona-1,3-diene: λ_{max} 213 mμ (ε 2300); P. D. Gardner, private communication. Cyclonona-1,3,6-triene: λ_{max} 223 mμ (log ε 3.61); W. R. Roth, *Ann.*, **671**, 10 (1964). Cycloocta-1,3-diene: λ_{max} 228 mμ (log ε 3.75); "Organic Electronic Spectral Data," Vol. III, Wheeler and L. A. Kaplan, Ed., Interscience Publishers, New York, N. Y., 1966, p 165.

(7) The Monsanto Co., St. Louis, Mo.