We have examined the selectivity of rat liver microsomal epoxide hydrolase (EC 3.3.2.3) toward all of the possible positional isomers of benzo-ring diol epoxides and tetrahydroepoxides of benz[a]anthracene, as well as the 1,2-diol 3,4-epoxides of triphenylene. This set includes compounds with no bay region in the vicinity of the benzo-ring, a bay-region diol group, a bay-region epoxide group, and (for the triphenylene derivatives) both a bay-region diol and a bay-region epoxide. In all cases where both the tetrahydroepoxides and the corresponding diol epoxides were examined, there is a large retarding effect of hydroxyl substitution on the rate of the enzyme-catalyzed hydration. When the tetrahydroepoxides are fair or poor substrates (epoxide group in the 1,2-, 8,9-, or 10,11-position), the additional retardation introduced by adjacent hydroxyl groups causes the enzyme-catalyzed hydrolysis of the corresponding diol epoxides to be insignificantly slow or nonexistent. In contrast, a benz[a]anthracene derivative with an epoxide group in the 3,4-position, (-)-tetrahydrobenz[a]anthracene (3R,4S)-epoxide, has been identified as the best substrate known for epoxide hydrolase, with a V_max at 37 °C and pH 8.4 of 6800 nmol/min/mg of protein, and the two diastereomeric (±)-benz[a]anthracene 1,2-diol 3,4-epoxides, unlike all the other diol epoxides examined to date, are moderately good substrates for epoxide hydrolase. This novel observation is accounted for by the fact that the very high reactivity of the tetrahydrobenz[a]anthracene 3,4-epoxide system towards epoxide hydrolase is large enough to overcome a kinetically unfavorable effect of hydroxyl substitution. The enantioselectivity and positional selectivity of the enzyme have been determined for the tetrahydro-1,2- and 1,3,4-epoxides of benz[a]anthracene as well as the 1,2-diol 3,4-epoxides. When the epoxide is located in the 3,4-position, the benzylic carbon is the preferred site of attack, whereas for the enantiomers of the bay-region tetrahydro-1,2-epoxides, the chemically less reactive nonbenzylic carbon is preferred. The regio- and enantioselectivity of epoxide hydrolase are discussed in terms of a possible model for the hydrophobic binding site of this enzyme.

Angular benzo-ring diol epoxides in which the epoxide moiety occupies a bay region of the hydrocarbon molecule have been shown to be important ultimately carcinogenic and mutagenic metabolites of a large number of polycyclic aromatic hydrocarbons (1). Metabolically these diol epoxides arise by cytochrome P-450-catalyzed epoxidation to form an arene oxide, epoxide hydrolase-catalyzed opening of the arene oxide to a trans-dihydrodiol, and subsequent cytochrome P-450-catalyzed epoxidation of the nonaromatic double bond. Significantly, all of the bay-region diol epoxides studied to date are resistant to hydrolytic inactivation by microsomal epoxide hydrolase (EC 3.3.2.3) (2–7), although in those cases that have been studied the corresponding bay-region tetrahydroepoxides which lack the hydroxyl groups (with the exception of tetrahydrobenz[a]acridine 1,2-epoxide (7)) are substrates for this enzyme (2–4). “Reverse” diol epoxides, in which the diol group occupies a bay region and the epoxide group does not, can also exist. Benzo[a]pyrene 9,10-diol 7,8-epoxide, the only such reverse diol epoxide that has been tested, is an extremely weak mutagen when compared with the bay-region epoxide, benzo[a]pyrene 7,8-diol 9,10-epoxide, and the corresponding 9,10-dihydrodiol is not tumorigenic (8).

In the present study we report the first examples of benzo-ring diol epoxides that are good substrates for epoxide hydrolase, namely the diastereomeric reverse 1,2-diol 3,4-epoxides derived from benz[a]anthracene, in which the benzylic hydroxyl group and the epoxide oxygen are cis (±-1a) and trans (±-2a) (Fig. 1).

We were first led to consider the possibility that these reverse diol epoxides of benz[a]anthracene were substrates for epoxide hydrolase by an observation made during investigation (9) of the metabolism of benz[a]anthracene 1,2-epoxide (7) which the diastereomeric reverse 1,2-diol 3,4-epoxides (to give tetraol products resulting from cis and trans hydration at the benzylic position of the epoxide) and epoxide hydrolase-catalyzed hydrolysis (to give only the trans hydration product). Since 1a is thus the first benzo-ring diol epoxide presumed to be a substrate for epoxide hydrolase, we have investigated...
Stereoselectivity of Epoxide Hydrolase

**EXPERIMENTAL PROCEDURES**

Materials—Syntheses of the racemic diol epoxides 1a and 2a and the enantiomeric tetrahydrobenz[a]anthracene 1,2-, 3,4-, and 8,9-epoxides (+)-3b and (−)-3b (10), (+)-3a and (−)-3a, (+)-3d and (−)-3d (12), as well as racemic 10,11-epoxide (±)-3c and 10,11-epoxide (±)-2c (14), were described elsewhere. Benz[a]anthracene 3,4-diol 1,2-epoxides (±)-1b and (±)-2b (14) and their enantiomers (11), 8,9-diol 10,11-epoxides (±)-1c and (±)-2c (14), 10,11-diol 8,9-epoxide (±)-2d (14), and triphenylene 1,2-diol 3,4-epoxides (±)-1e and (±)-2e (15) were synthesized as described. Substrates were shown to be free of impurities by NMR spectroscopy. For all diol epoxide-1 isomers, the benzylic hydroxyl group and the epoxide oxygen have cis relative stereochemistry whereas these groups are trans in the diol epoxide-2 diastereomers. Tetraols derived upon nonenzymatic hydrolysis of (±)-1a and 2a (9) and (±)-1b and 2b (16) have also been described. In this nonenzymatic hydrolysis, attack of water occurs only at the benzylic position. Purified rat liver microsomal epoxide hydrolase was obtained from isosafrole-treated rats as described (17) and had a specific activity of 1294 nmol/min/mg of protein with octene 1,2-oxide as substrate. A single enzyme preparation was used throughout the study. To ensure internal consistency of results the activity of the preparation was periodically verified by measurement of the rate with (+)-tetrahydrobenz[a]anthracene 3,4-epoxide (+)-3a (1.45 μM), an excellent substrate that exhibited linear kinetics at this substrate concentration (see below).

Enzyme Kinetics—All enzymatic reactions were carried out at 37 °C in 0.05 M Tris-HCl buffer containing 0.1 M sodium chloride, at pH 8.37 ± 0.03 (measured at 37 °C), the optimal pH value for enzymatic hydrolysis of (±)-1a. Substrates were dissolved in acetonitrile. Where necessary for substrate stabilization small amounts of ammonium hydroxide (51%) were added to these stock solutions. Reactions were initiated by addition of the substrate solution to a cuvette containing the buffered enzyme solution at 37 °C, to give a final acetonitrile concentration of 0.5% for the diol epoxides (with the exception of (±)-2d, for which the final acetonitrile concentration was 1%) or 2% for the tetrahydroepoxides. In all experiments where substrate concentrations were varied, compensating amounts of acetonitrile were added to give identical acetonitrile concentrations throughout the series of determinations for that substrate. Concentrations of acetonitrile up to 2% were shown not to affect the activity of the enzyme towards (±)-1a. In the case of (±)-2e, the substrate was added as a 5 mM solution in dimethyl sulfoxide, to give a final concentration of 0.2% dimethyl sulfoxide. Kinetics of the reactions were followed, using a Cary 219 spectrophotometer, at 261 nm (compounds (±)-1a, (±)-1b, (±)-1c, (±)-2b, (±)-2c, and (±)-2d), 260 or 261 nm (±)-3b and (±)-3b, 254 nm (±)-1c and (±)-2e, 302.5 nm (±)-3c, 298 nm ((±)-2d), (±)-3d, and (±)-3d, and 306 nm (±)-1e and

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2 H. Yagi and D. M. Jerina, manuscript in preparation.

3 D. R. Boyd, H. Yagi, and D. M. Jerina, unpublished work. The method for synthesis of (±)- and (−)-3a was essentially that described for the enantiomeric 1,2,3,4-tetrahydrochrysene 3,4-epoxides (Ref. 11).
Upon complete acid hydrolysis of 1, their absolute configurations have been deduced in the present study with the values determined from the initial rates of the enzymatic reaction.

Diol epoxides and tetrahydroepoxides. Signs of rotation are not given for the diol epoxides as they have not been resolved chemically, (+)-3e).

The pseudo-first-order rate constant for uncatalyzed hydrolysis, to the concentration at time zero, was applied (see equation). In the presence of 1.0 ml of 2 M mercaptoethanol solution containing 0.8 mol fraction as the sodium salt. The reaction mixture was cooled and was measured by cis hydration (k" = 2.5) and the thioether adduct of 2a (k" = 3.5) by preparative HPLC on a Du Pont Zorbax ODS column (4.6 x 250 mm) eluted with a linear gradient (1%/min) of 22-42% acetonitrile in water at a flow rate of 1 ml/min.

Products of Enzymatic Hydrolyses—The hydrolyses of racemic diol epoxides 1a and 2a (16.5 µmol) were allowed to proceed to completion in the presence of 12 µg/ml of epoxide hydrolase in 30 ml of the Tris-NaCl buffer described above. After completion of reaction as determined by UV the incubation mixtures were extracted three times with 2-ml portions of ethyl acetate. The ethyl acetate extracts were evaporated to dryness and the residue was dissolved in acetonitrile and subjected to high performance liquid chromatography (HPLC) on a Du Pont Zorbax ODS column (4.6 x 250 mm) eluted with a linear gradient (1%/min) of 22-42% acetonitrile in water at a flow rate of 1 ml/min.

To determine the enantiomeric selectivity of the enzyme, 78 µg of epoxide hydrolase and 50 µmol of racemic 2a were allowed to react at 37 °C in 30 ml of buffer, until approximately 1/2 of the total absorbance change had occurred (~800 s). The mixture was quenched by addition of 1.0 ml of 2 M mercaptoethanol solution containing 0.8 mol fraction as the sodium salt. The reaction mixture was cooled and extracted three times with 10-ml portions of ethyl acetate and the ethyl acetate extracts were washed with 1 M sodium carbonate and water, dried over sodium sulfate, and evaporated. The product (tetraol trans-2; cf. Scheme I) resulting from benzylic trans hydration (k'^{-1}) was separated from a small amount of tetraol cis-hydrolase, which was determined by cis hydration (k" = 2.5) and the thioether adduct of 2a (k" = 3.5) by preparative HPLC on a Du Pont Zorbax ODS column (4.6 x 250 mm) eluted with 65% methanol in water at a flow rate of 2 ml/min.

A similar procedure was employed with racemic 1a; in this case the reaction was stopped after 21 min with 2.0 ml of the mercaptoethanol reagent described above and 0.375 ml of 1 M sodium hydroxide. After extraction and workup, the tetraol trans-1 (Scheme I) (k" = 1.1) was separated from the thioether adduct (k'^{-1} = 2.1) by preparative HPLC as described using 70% methanol in water as the eluent at a flow rate of 1.6 ml/min.

Circular dichroism spectra of the two enzymatically derived tetraols from 1a and 2a were measured using a JASCO J500A spectropolarimeter. Reference tetraols of known absolute configuration were prepared by acid hydrolysis (which proceeds exclusively by benzylic hydration) of the bay-region epoxides (+)-(3S,4R)-dihydroxy-(1R,2S)-epoxy-1,2,3,4-tetrahydrobenz[a]anthracene (dihydro epoxide 1b) and (+)-(3R,4S)-dihydroxy-((1S,2R)-epoxy-1,2,3,4-tetrahydrobenz[a]anthracene (dihydro epoxide 2b) in 19-diozone/water containing 1 mM perchloric acid and 0.1 M sodium perchlorate. Under these conditions the half-times for hydrolysis of 1b and 2b are 6 and 2.2 min, respectively (20). The tetracols resulting from the trans opening of the epoxide were separated from the isomeric tetracols resulting from cis opening by preparative HPLC (70% methanol in water) as described. In both cases the tetracols resulting from trans opening are the more polar isomers.

\[ \text{The abbreviation used is: HPLC, high performance liquid chromatography.} \]
treatment at room temperature (16–24 h) with pyridine and (−)-menthylloxazacetyl chloride, followed by addition of benzene and extractive workup. The resultant mixtures of bis-menthylxyacetates were analyzed by HPLC on a Du Pont Golden SIL column eluted with 7.5% ether in cyclohexane at a flow rate of 2.2 ml/min. The following k' values were observed for the bis-[(−)-menthylxyacetates] of the four trans-tetrahydrodiols: (3R,4R), 4.3; (3S,4S), 5.6; (1R,2R), 6.1; and (1S,2S), 8.4. Correction for the (1S,2S)-diol formed from (+)-(1R,2S)-3b and for the (1R,2R)-diol formed from (−)-(1S,2R)-3b by competitive nonenzymatic hydrolysis was made based on the observation of 15 and 28% cis-diol in the hydrolysis mixtures of (−)-3b and (−)-3b, respectively. Control reactions containing the same amount of enzyme and 7.6 μM (−)-3b or (−)-3b, run in the presence of the inhibitor 1,2-epoxy-3,3,3-trichloropropene (790 μM, 100% ratio with substrate), gave cis/trans ratios of 1:3.1:0 (for the (+)-epoxide) and 1:15:1.0 (for the (−)-epoxide). Thus, 13.5 and 34% of the total trans-diol from (+)-3b and (−)-3b, respectively, must have been nonenzymatically formed and must be the enantiomer resulting from benzyl attack.

RESULTS

Fig. 3 shows the dependence of the observed initial rates for enzymatic hydrolysis on the concentration of (±)-benz[a]anthracene 1,2-diol 3,4-epoxides 1a and 2a. Apparent Kₘ and Vₘₐₓ values for the two diastereomers (Table II) are comparable and are in the range expected for moderately good substrates for this enzyme. Under similar conditions (13–16 μg of protein/ml) no enzymatic reaction was detectable with either diastereomer of benz[a]anthracene 3,4-diol 1,2-epoxide ((±)-1b and (±)-2b) as will be shown later. 1,2-Epoxy-3,3,3-trichloropropene, a potent inhibitor of microsomal epoxide hydrolase (21), at a concentration of 33 μM, gave 97% inhibition of enzymatic hydrolysis of 12.5 μM (±)-1a.

Chromatographic analysis (cf. Fig. 4) of the enzymatically formed products from (±)-1a and (±)-2a demonstrated that, from each diastereomer, tetraols were formed by trans addition of water to the diol epoxides. In each case the enzymatic product was chromatographically identical to the tetraol obtained from the same diastereomer by acid-catalyzed trans opening of the epoxide, and no trace of the tetrol formed by trans opening of the other diastereomeric epoxide was detected. Acid hydrolysis is known to give only products of benzyl C-O cleavage. Thus, the enzymatic reaction must also occur exclusively by attack of water on the benzyl carbon atom (Scheme I).

At low initial substrate concentrations, where the enzymatic reaction of each enantiomer is pseudo-first-order, the reactions of racemic 1a and 2a exhibit biphasic kinetics when carried to completion, as anticipated if the enzyme is enantioselective (Fig. 5). The observed pseudo-first-order rate constants, k' and k", for the individual enantiomers in a given racemic mixture under these conditions are given by k' = (k₀ + [E]Vₘₐₓ/Kₘ') and k" = (k₀ + [E]Vₘₐₓ/Kₘ") where k₀ is the rate constant for hydrolysis in solvent alone and [E] is the concentration of the enzyme. The apparent values of Kₘₐₓ and Vₘₐₓ determined from initial rate studies with the racemic substrates are related to the individual kinetic constants for each enantiomer (Vₘₐₓ and Kₘ) by Equations 1 and 2. Thus, Vₘₐₓ/Kₘₐₓ for each racemic substrate

\[ K_{\text{app}} = 2k'K_m'K_m''/(K_m' + K_m'') \]

(1)

\[ V_{\text{max}}' / K'_m = [V_{\text{max}} (K_m' + V_{\text{max}} (K_m') + K_m') / (K_m' + K_m'') ] \]

(2)

is related by Equations 3, 4a, and 4b to the pseudo-first-order rate constants k' and k":

\[ V_{\text{max}} / K_m = \left( V_{\text{max}} (K_m' + V_{\text{max}} (K_m') + K_m') / 2 \right) \]

(3)

\[ V_{\text{max}} / K_m = (k' - k_0)/[E] \]

(4a)

\[ V_{\text{max}}(K_m' + K_m') = (k'' - k_0)/[E] \]

(4b)

Equation 3 and values of Vₘₐₓ/Kₘ, and Vₘₐₓ/Kₘ (last column

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Total substrate</th>
<th>Total volume</th>
<th>Final concentration CH3CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-3a</td>
<td></td>
<td>195 μmol</td>
<td>0.11</td>
<td>38</td>
</tr>
<tr>
<td>(+)-3a</td>
<td></td>
<td>23</td>
<td>0.10</td>
<td>51</td>
</tr>
<tr>
<td>(+)-3b</td>
<td></td>
<td>470</td>
<td>0.33</td>
<td>3.3</td>
</tr>
<tr>
<td>(+)-3b</td>
<td></td>
<td>310</td>
<td>0.045</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Added in equal portions.
* Added in 10 equal portions.
* Added in four 5-μl and seven 10-μl portions.

Preparative-scale enzymatic reactions for the identification of products from the optically active tetrahydroepoxides, 3a and 3b, of benz[a]anthracene were carried out as outlined in Table I. In the instances indicated, where low volumes and high enzyme concentrations were required, the substrate was added in several successive small portions to avoid possible precipitation of the poorly soluble epoxides. In these cases the reaction was followed spectrophotometrically, and additional substrate was added after all or most of the previous portion of substrate had reacted. After completion of reaction, the products were extracted into ethyl acetate which was then washed with saturated sodium chloride solution and dried with sodium sulfate. The products from the tetrahydro-3,4-epoxides were exclusively trans-3,4-diols as shown by HPLC, whereas spontaneous hydrolysis of the 1,2-epoxides competes with enzymatic hydrolysis leading to contamination of the trans-diol products by cis-diols. The mixture of enantiomeric trans-diols from each enantiomer of the 1,2-epoxide was isolated as the more polar component upon preparative HPLC on a Du Pont Zorbax ODS column (9.4 × 250 mm) eluted with 80 or 85% methanol in water at a flow rate of 2 ml/min. The trans-diols were converted to their bis-[(−)-menthylxyacetates] (10, 11) by

### Scheme I

**Path A**, benzyl attack; **Path B**, non-benzyl attack.
FIG. 3. Dependence on substrate concentration (<10% of reaction) of hydrolysis of racemic 1a and 2a by epoxide hydrolase (13 μg/ml). Kinetic conditions are described in the text. Lineweaver-Burk plots are shown as insets. The curves are based on the following constants: (+)-1a, $K_m = 16.4 \mu M$, $V_{max} = 6.3$ nmol of substrate/s/mg of protein; (+)-2a, $K_m = 20 \mu M$, $V_{max} = 4.3$ nmol of substrate/s/mg of protein.

![Graph of substrate concentration vs. hydrolysis rate for 1a and 2a.]

### Table II

**Kinetic constants for hydration of substrates by epoxide hydrolase**

Rates of reaction were measured spectrophotometrically at pH 8.37 and 37 °C as described in the text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Protein</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/s/mg protein)</th>
<th>$V_{max}/K_m$ (s⁻¹/mg protein/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-1a</td>
<td>1.3-13</td>
<td>14.0 ± 3.6ᵃᵇ</td>
<td>5.2 ± 1.4ᵃᵇ</td>
<td>0.51⁽⁶⁾ (3R,4S)($V_{max}/K_m$)</td>
</tr>
<tr>
<td>(±)-2a</td>
<td>13</td>
<td>20ᵃ</td>
<td>4.3ᵃ</td>
<td>0.05³ (3S,4R)($V_{max}/K_m$)</td>
</tr>
<tr>
<td>(+)-3a (3S,4R)</td>
<td>2.6</td>
<td>0.10</td>
<td>2.3</td>
<td>0.36⁽⁶⁾ (3S,4R)($V_{max}/K_m$)</td>
</tr>
<tr>
<td>(±)-3b (1R,2S)</td>
<td>0.52</td>
<td>1.4</td>
<td>113</td>
<td>0.086⁽³⁾ (3R,4S)($V_{max}/K_m$)</td>
</tr>
<tr>
<td>(±)-3c</td>
<td>15.6</td>
<td>0.6</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>(+)-3d (8R,9S)</td>
<td>3.9, 15.6</td>
<td>0.5</td>
<td>1.3</td>
<td>0.06-0.16ᵈ</td>
</tr>
<tr>
<td>(±)-3d (8S,9R)</td>
<td>3.9, 7.8</td>
<td></td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Average (±S.D.) of 3 determinations at 3 protein concentrations.
ᵇ From observed biphasic pseudo-first-order kinetics for hydrolysis of the racemic substrates. A correction for nonenzymatic hydrolysis ($k_b = 5.6 \times 10^{-4} s^{-1}$) was applied to the observed pseudo-first-order rate constants for 2a. Assignment of the more reactive enantiomers was accomplished by circular dichroism spectroscopy of the product isolated after ~50% reaction (see text).
ᶜ From observed pseudo-first-order kinetics for hydrolysis of the racemic substrates (4 μM). Complete reaction of the substrate was observed. Pseudo-first-order kinetics were linear, with experimental error, for ~90% of the reaction. This observation suggests that the rate constants for the two enantiomers are no more than 2- to 3-fold different from each other, as indicated by the upper and lower limits given for values of these individual rate constants.

d of Table II) determined from $k'$ and $k''$ for the pseudo-first-order kinetics can be used for independent calculation of $V_{max}/K_m^{exp}$, to verify the values determined from initial rate measurements. Calculated values of $V_{max}/K_m^{exp}$ of 0.28 and 0.22 s⁻¹/mg of protein/ml for (±)-1a and (±)-2a, respectively, are in acceptable agreement with the values of 0.37 and 0.21 s⁻¹/mg of protein/ml from the initial rate experiments.

The tetraol formed from the preferred enantiomer of each substrate was isolated after trapping the unreacted epoxide after ~50% reaction. The configurations of these tetraols were ascertained by comparison of their circular dichroism spectra with spectra of the tetraols produced by acid hydrolysis of enantiomerically pure benz[a]anthracene 3,4-diol 1,2-epoxides (Scheme 2). The signs of the ellipticity observed for the tetraols trans-1 and trans-2 derived from (+)-1b and (−)-2b, respectively, are in agreement with those reported for major tetraol products from the microsomal metabolism of the (+)-(1S,2S)- and (−)-(1R,2R)-dihydriodiols of benz[a]anthracene, respectively (9, 22). These configurational assignments, together with our previous determination that the exclusive site of enzymecatalyzed ring cleavage is at the benzylic carbon, C-4, established that the major tetraol derived from racemic 1a at low substrate conversion is (1R,2S,3S,4R), and the tetraol derived from racemic 2a is (1R,2S,3R,4S). Thus, epoxide hydrolase preferentially attacks the (1R,2S,3R,4S)-enantiomer of 1a and the (1R,2S,3S,4R)-enantiomer of 2a (Scheme 2 and Fig. 2). Kinetic constants for the hydration of substrates (±)-1a and (±)-2a as well as the enantiomerically pure tetrahydroepoxides (+)-3a, (−)-3a, (+)-3b, and (−)-3b are summarized in Table II.
The availability of enantiomerically pure tetrahydroepoxides $3a$ and $3b$ of known absolute configuration (Fig. 2) (10, 11) enabled us to determine the positional specificity of epoxide hydrolase from the configurations of the enzymatically formed trans-diols, since trans-diol formation must occur with inversion of configuration at the carbon attacked by water. Configurations of the diol products were established by cochromatography of their bis[(+)-menthyl]acetate esters with the bis[(−)-menthyl]acetates of known (10, 11) absolute configuration (Table III).

Attack at the more reactive benzylic carbon predominates for both (+)-3a and (−)-3a. However, the bay-region epoxides (+)-3b and (−)-3b exhibit a reversal of this trend, indicating that in this case steric factors are large enough to overcome the anticipated large electronic preference for benzylic attack.

In order to delineate further the specificity of epoxide hydrolase towards diol epoxides and tetrahydroepoxides, we have investigated examples of all possible positional isomers of benzo-ring tetrahydroepoxides and diol epoxides derived from benz[a]anthracene as potential substrates for the enzyme. These include the diastereomeric benz[a]anthracene 8,9-epoxide 10,11-epoxides (±)-1c and (±)-2c and the tetrahydro-10,11-epoxide (±)-3c, one diastereomer in the 10,11-diol 8,9-epoxide series, (±)-2d, and the corresponding enantiomeric tetrahydro-8,9-epoxides (+)-3d and (−)-3d. We also examined the triphenylene diol epoxides (±)-1e and (±)-2e. The latter compounds were chosen for investigation because their preferred conformations in solution resemble those of the diol epoxides (±)-1a and (±)-2a in that both diastereomers (±)-1a and (±)-2a, like (±)-1a and (±)-2a, exhibit a stronger preference for the diaxial conformation of the diol group than do analogous diol epoxides with a bay-region epoxide and a non-bay-region diol group. Of these compounds the (+)- and (−)-tetrahydrobenz[a]anthracene 8,9-epoxides, (+)-3d and (−)-3d, are 10- to 80-fold poorer substrates (based on $V_{max}/K_m$) than (+)-3a and (−)-3e, whereas (±)-3c is an even poorer substrate by an additional factor of 10–20 (Table IV). All of the diol epoxides were very poor substrates for epoxide hydrolase (Table IV) and were not further investigated.

**DISCUSSION**

The reverse diol epoxides (±)-1a and (±)-2a represent the first examples of benzo-ring diol epoxides (derived from polycyclic aromatic hydrocarbons) that are good substrates for microsomal epoxide hydrolase. Values of $V_{max}$ of 4–5 nmol/s/mg of protein for racemic 1a and 2a (240–300 nmol/min/mg of protein) are comparable with the values of 445 nmol/min/mg of protein and 500–685 nmol/min/mg of protein observed for benz[a]pyrene 4,5-oxide and styrene oxide, respectively, both moderately good substrates for this enzyme (23). In contrast, no enzymatic catalysis is detectable for the bay-region diol epoxides (±)-1b and (±)-2b, as was previously reported using mutagenic activity as the method of endpoint analysis (3). The failure of epoxide hydrolase to catalyze the hydrolysis of bay-region diol epoxides in general (2–7) has important consequences biologically, since the absence of this detoxification route means that bay-region epoxide diols are more likely to exist long enough in the cellular milieu to react with essential macromolecules, initiating a toxic, mutagenic or tumorigenic process. The tetrahydrobenz[a]anthracene 3,4-epoxides are unique among the benzo-ring tetrahydroepoxides of benz[a]anthracene in their extraordinarily high reactivity ($V_{max}/K_m$) with epoxide hydrolase. In comparison, the tetrahydrobenz[a]anthracene 8,9-epoxides are only fair substrates for epoxide hydrolase, whereas the tetrahydrobenz[a]anthracene 1,2- and 10,11-epoxides are very poor substrates. Comparison of the diol epoxides and tetrahydroepoxides indicates that in all cases the diol epoxides are much poorer substrates than the corresponding tetrahydroepoxides. Introduction of hydroxyl groups (to give benzo-ring diol epoxides) retards the enzymatic reaction to such an extent that enzymatic reaction of the corresponding diol epoxides is negligibly slow (see below) when the epoxide group is in the 1,2-, 8,9-, or 10,11-position. However, because of the extraordinarily high reactivity of the tetrahydro-3,4-epoxides, the corresponding diol epoxides (±)-1a and (±)-2a are still moderately good substrates for epoxide hydrolase even with the substantial rate
retardation caused by hydroxyl substitution. Hence, our observation that these reverse diol epoxides, unlike all other diol epoxides investigated to date, are good substrates for epoxide hydrolase is a consequence of the regioselectivity of the enzyme.

Tetrahydroepoxides—Within the present series of benz[a]anthracene derivatives (Table II), the best substrates (based on $V_{\text{max}}/K_m$) are the non-bay-region tetrahydroepoxides (+)-3a and (-)-3a. The tetrahydroepoxide (-)-3a has the highest value of $V_{\text{max}}$ (113 nmol/s/mg of protein or 6800 nmol/min/mg of protein) of 17 substrates examined to date (23-28). Indirect assays using mutagenic activity for endpoint analysis also indicate that (-)-3a is an exceptionally good substrate (29). The enantiomeric epoxide, (+)-3a, although exhibiting only a moderate $V_{\text{max}}$, has an exceptionally low value of $K_m$. The tetrahydro-8,9-epoxides (+)-3d and (-)-3d are only fair substrates for epoxide hydrolase. $V_{\text{max}}$ for the (+)-enantiomer is 1.26 nmol/s/mg of protein (76 nmol/min/mg of protein), and although $V_{\text{max}}$ was not determined for the other enantiomer due to its higher $K_m$ and the limited solubility of these compounds, values of $V_{\text{max}}/K_m$ for the two enantiomers are comparable. In the case of both the enantiomeric pairs of tetrahydro-3,4- and tetrahydro-8,9-epoxides, the (+)-enantiomer enantiomer, in which the chirality of the benzylic carbon is $(R)$, exhibits the smaller value of $K_m$ and also the smaller value of $V_{\text{max}}$. (Although $K_m$ and $V_{\text{max}}$ were not measured for (-)-3d, no deviation from pseudo-first-order kinetics was observed for the reaction of this compound at a concentration of 5.6 $\mu$M, indicating that $K_m$ must be >5 $\mu$M and thus $V_{\text{max}}$ must be >5 nmol/s/mg of protein).

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Less polar bis[(-)-menthyl-oxacycetate]</th>
<th>Benzylic attack</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-3a  (3S,4R)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>(-)-3a  (3R,4S)</td>
<td>&gt;95</td>
<td>&gt;95*</td>
</tr>
<tr>
<td>(+)-3b  (1R,2S)</td>
<td>86</td>
<td>14</td>
</tr>
<tr>
<td>(-)-3b  (1S,2R)</td>
<td>12*</td>
<td>12*</td>
</tr>
</tbody>
</table>

*For the trans-3,4-diols (from 3a) the less polar bis[(-)-menthyl-oxacycetate] diastereomer is (3R,4R) and for the trans-1,2-diols (from 3b) the less polar bis[(-)-menthyl-oxacycetate] is (1R,2R) (Refs. 10 and 11).

A peak in the chromatogram with $k'$ similar to the more polar menthyl-oxacycetate, and corresponding to <5% of the major peak was observed; as the quantity was insufficient for spectral identification, the identity of this material is unknown.

*Corrected for trans-diol which was formed nonenzymatically; see text.

It is of interest to compare these results with the relative values of $K_m$ and $V_{\text{max}}$ observed for the enantiomeric benzo[a]pyrene 7,8-oxides (30). Fig. 6 shows the structures of (+)-3a, (+)-3d, and (+)-benzo[a]pyrene (7R,8S)-oxide, as well as the enantiomeric series (-)-3a, (-)-3d, and (-)-benzo[a]pyrene (7S,8R)-oxide, aligned so that their epoxide groups would overlap if the molecules were superimposed. It is apparent...
TABLE IV
Hydration of diol epoxides that are "poor" substrates for epoxide hydrolase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme concentration</th>
<th>Substrate concentration</th>
<th>Initial rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg protein/ml</td>
<td>µM</td>
<td>nmol/s/mg protein</td>
</tr>
<tr>
<td>Benz[a]anthracene 3,4-diol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2-epoxide</td>
<td>15.6</td>
<td>17.5</td>
<td>≤0.3</td>
</tr>
<tr>
<td>(±)-1b</td>
<td>13.0</td>
<td>7.5</td>
<td>≤0.08</td>
</tr>
<tr>
<td>Benz[a]anthracene 8,9-diol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,11-epoxide</td>
<td>15.6</td>
<td>12</td>
<td>0.03</td>
</tr>
<tr>
<td>(±)-1c</td>
<td>15.6</td>
<td>10</td>
<td>0.05</td>
</tr>
<tr>
<td>Benz[a]anthracene 10,11-diol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,9-epoxide</td>
<td>15.6</td>
<td>13</td>
<td>0.03</td>
</tr>
<tr>
<td>(±)-2d</td>
<td>15.6</td>
<td>11</td>
<td>0.03</td>
</tr>
<tr>
<td>Triphenylene 1,2-diol 3,4-epoxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-1e</td>
<td>15.6</td>
<td>10</td>
<td>≤0.02</td>
</tr>
<tr>
<td>(±)-2e</td>
<td>15.6</td>
<td>10</td>
<td>≤0.02</td>
</tr>
</tbody>
</table>

Fig. 6. Comparison of K<sub>m</sub> and V<sub>max</sub> values for related enantiomeric pairs of substrates for epoxide hydrolase. Units for K<sub>m</sub> values are µM and those for V<sub>max</sub> values are nmol/s/mg of protein. Kinetic constants for the benzo[a]pyrene (B[a]P) 7,8-oxide enantiomers cannot be directly compared with those for the tetrahydrobenz[a]anthracene epoxides since the former were determined (Ref. 30) at 25 °C, and in the presence of a detergent which increases the apparent K<sub>m</sub>, but the values for the two enantiomers in each set can be compared with each other. Although K<sub>m</sub> and V<sub>max</sub> for (−)-B[a]P 7,8-oxide were not determined with purified epoxide hydrolase, V<sub>max</sub> for this enantiomer was found to be ～1 times greater than V<sub>max</sub> for the (+)-oxide with a microsomal protein preparation. The broken lines represent a hypothetical hydrophobic binding site of epoxide hydrolase.

that the orientation of the polycyclic aromatic moiety in each series is similar. For each pair of enantiomers, the (+)-enantiomer exhibits the smaller value of K<sub>m</sub>. One interpretation of the low K<sub>m</sub> is that for these (+)-enantiomers, the aromatic portion occupies a proposed hydrophobic pocket of the enzyme that lies behind and to the right rear of the epoxide group when the molecule is oriented as shown in Fig. 6. The location of this hydrophobic pocket on the right is suggested by the observations of Armstrong et al. (31) on the enantioselectivity of epoxide hydrolase toward benzo[a]pyrene 4,5- and 7,8-oxides and benz[a]anthracene 5,6-oxide. This hydrophobic site also corresponds to the area that is preferentially occupied by the tert-butyl group of 4-tert-butyl-1,2-epoxycyclohexanes (32). Our present results suggest that the area behind (and possibly slightly to the left) of the epoxide is also available for substrate (and transition-state) binding on the basis of the relatively small value of K<sub>m</sub> and large value of V<sub>max</sub>/K<sub>m</sub> for (−)-(3R,4S)-3a (cf. Fig. 6).

Presumably because of their more favorable orientation relative to this proposed binding site, the (+)-enantiomers of 3a, 3d and benzo[a]pyrene 7,8-oxide exhibit lower values of K<sub>m</sub> than the corresponding (−)-enantiomers. In all three cases, the larger value of V<sub>max</sub> is exhibited by the opposite (−)-enantiomer. For (−)-3a and (−)-3d, the chemically more reactive benzylic carbon also has the (S)-chirality commonly preferred (31) in substrates of this type by epoxide hydrolase. The fact that these two factors coincide may account for the faster reaction rate. This explanation does not, however, account for the larger V<sub>max</sub> exhibited by (−)-benzo[a]pyrene (7S,8R)-oxide, since in this case the chemically more reactive position (and the position that is attacked by the enzyme) is the allylic (8R)-position. In the case of 3a, but not 3d or benzo[a]pyrene 7,8-oxide, V<sub>max</sub>/K<sub>m</sub> is larger for the (−)-enantiomer, as a result of the fact that the enhancement of V<sub>max</sub> for (−)-3a relative to (+)-3a (~50-fold) more than compensates for its less favorable K<sub>m</sub> (~10-fold).

Relative values of V<sub>max</sub>/K<sub>m</sub> for reaction at the benzylic and nonbenzylic positions of the enantiomers of 3a and 3b were calculated by multiplication of the observed values of V<sub>max</sub>/K<sub>m</sub> by the fraction of attack at each position (Table III) and are shown in Fig. 7. The observation that attack at C-3 occurs to the extent of ~20% with (+)-(3R,4S)-3a, but is undetectable with (−)-(3R,4S)-3a, provides a demonstration of the enzyme's tendency to attack at a center of (S)-chirality in these molecules, even when such attack is unfavorable based on chemical reactivity. The bay-region epoxide enantiomers, 3b, are poor substrates for epoxide hydrolase. Based on relative values of V<sub>max</sub>/K<sub>m</sub> for reaction at each position, the bay-region epoxides (+)-3b and (−)-3b are 10<sup>−4</sup>–10<sup>−14</sup> times less reactive toward enzymatic attack at the benzylic position than are the corresponding non-bay-region epoxides, (+)-3a and (−)-3a, whereas enzyme catalyzed non-benzylic attack on (+)-3b is only ~50-fold slower than attack at the analogous position of (−)-3a. Thus, these bay-
region epoxides undergo attack predominantly at the non-
benzylic position, even when benzylic attack is at a favored
(S)-center. The 10,11-epoxide, (+)-3c, is also a very poor
substrate for epoxide hydrolase. Since both this compound
and the 1,2-epoxide enantiomers, when oriented as in Figs. 6
and 7, have part of the aromatic moiety extending in front
of the postulated binding site, it is tempting to speculate that
this part of the enzyme is an unfavorable region for
substrate binding. Thus, the 1,2-epoxides, (+)-3b and (-)-3b,
may have to bind to the enzyme in an orientation with the
aromatic rings rotated somewhat to the rear of their position
shown in Fig. 7. Such an orientation could possibly result in
the altered positional selectivity for nucleophilic attack that
is observed with these epoxides.

**Diol Epoxides**—Benzo-ring diol epoxides metabolically
formed from trans-dihydriodols can exist as either of two
diastereomers, namely, the diastereomer in which the benzylic
hydroxyl group and the epoxide oxygen are cis, (isomer-1
series) and the diastereomer in which these groups are trans
(isomer-2 series). For each of these diastereomers, two enan-
tiomers are possible. By product studies and pseudo-first-
order kinetic measurements we have determined the values
of $V_{\text{max}}/K_m$ for these four isomers of benz[a]anthracene 1,2-
diol 3,4-epoxide (Table II). The products of enzymatic hy-
drolysis of all four isomeric diol epoxides result exclusively
from attack at the benzylic carbon. This finding made possible
the identification, by product isolation at ~50% substrate
conversion, of the more reactive enantiomer from each of the
two racemic diol epoxides, (+)-1a and (+)-2a. Values of $V_{\text{max}}/K_m$
are at least 10- to 200-fold lower than those for the corresponding
tetrahydroepoxides at both (3R,4S)-series and the
(3S,4R)-epoxide series, whereas this rate retardation for the
enantiomers of isomer-2 is ~900-fold in the (3R,4S)-epoxide
series but only ~50-fold in the (3S,4R)-epoxide series. This
behavior of isomer-2 results in an observed reversal of the
expected preference for attack at an (S)-benzylic center in
the case of the two enantiomeric isomers-2, i.e. the (3S,4R)-
enantiomer reacts about three times faster (Table II) than the
(3R,4S)-enantiomer, although both are attacked exclu-
sively at C-4.

These relative reactivities can be interpreted either as an
especially low reactivity or poor fit to the enzyme of
(1S,2R,3S,4S)-2a or an especially high reactivity or good fit
to the enzyme of (1R,2S,3R,4R)-2a. The (3R,4S)-epoxide series
shows the hydroxyl groups are located in the hydrophobic site
to the right of the epoxide group (cf. Fig. 6), and hence the
enzyme may be particularly sensitive to their position and
orientation. This could conceivably result in an especially
poor fit for (1S,2R,3S,4S)-2a. Alternatively or additionally,
conformational factors may play a role in the different relative
rates for isomer-2 in the two enantiomeric series. Because of
their position in a hindered bay region, the hydroxyl groups
in both 1a and 2a prefer a pseudoaxial orientation (Fig. 8).
In the case of 1a, this corresponds to a conformation of the
tetrahydro benzo-ring (the "nonaligned" conformation) in
which the benzylic C-O bond of the epoxide is not aligned
with the $\pi$ orbitals of the benzene rings (20, 33). In the case of
2a, pseudoaxial orientation of the hydroxyl groups causes
the tetrahydro benzo-ring to assume the other possible con-
formation ("aligned") in which the benzylic C-O bond of the
epoxide is more nearly parallel with the $\pi$ orbitals of the
aromatic system. The aligned conformation is the more re-
active in pH-independent hydrolysis and/or rearrangement

Fig. 8. Illustration of the preferred conformations of (1S,2R,3S,4R)-1a and (1R,2S,3R,4R)-2a showing the tilting of the aromatic moiety in the preferred conformation of 2a when the epoxide groups are identically oriented. The structures shown were computer-generated based on representative x-ray structures for both conformations of the tetrahydro benzo-ring (Refs. 33 and 40).
reactions (20, 33). Thus, 2a, which prefers the aligned conformation, undergoes pH-independent hydrolysis and rearrangement in 1:9 dioxane:water ~17 times more rapidly than 1a. Furthermore, the reaction of (±)-2a with mercaptoethanol anion in 10% dioxane in water, which presumably occurs by nucleophilic attack upon the epoxide, is 2.5 times faster than the corresponding reaction of (±)-1a (data not shown). In contrast, the isomer-2 diastereomers of non-bay-region diol, bay-region epoxides which prefer the nonaligned conformation, undergoes pH-independent hydrolysis and rearrangement toward both pH-independent hydrolysis (20, 34, 35) and nucleophilic attack by thiolate anions (36, 37).

The corresponding tetrahydroepoxides appears to be the overriding factor in determining whether a diol epoxide will be a substrate for epoxide hydrolase. As previously demonstrated, extreme susceptibility of the epoxide moiety at a given position to enzymatic attack (as shown by high reactivity of the corresponding tetrahydroepoxides) appears to be the overriding factor in determining whether a diol epoxide will be a substrate for epoxide hydrolase. The specific factors that determine which tetrahydroepoxides will have such “superreactivity” with epoxide hydrolase, and consequently which, if any, other diol epoxides will be substrates, remain to be investigated.

REFERENCES


Stereoselectivity of Epoxide Hydrolase