Stereoselectivity of Isozyme C of Glutathione *S*-Transferase toward Arene and Azaarene Oxides†

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ABSTRACT: Three of the isozymes of glutathione *S*-transferase (EC 2.5.1.18) from rat liver (isozymes A, B, and C) catalyze the addition of glutathione to phenanthrene 9,10-oxide with varying degrees of efficiency and stereoselectivity. Isozyme C is 2-fold and 35-fold more efficient toward this substrate than are isozymes A and B, respectively, and gives a 20 to 1 ratio of the two possible diastereomeric products. The stereoselectivities of isozymes A (~1 to 1) and B (3 to 1) are considerably lower. The major product diastereomer from isozyme C is deduced to have the 9*S*,10*S* absolute configuration by circular dichroism spectroscopy, implying attack of glutathione on the oxirane carbon on *R* absolute configuration. Isozyme C shows little kinetic discrimination between other K-region arene oxides such as pyrene 4,5-oxide and the enantiomers of benz[*a*]anthracene 5,6-oxide and benzo[*a*]pyrene 4,5-oxide. However, the stereoselectivity toward all the substrates is conserved with predominant (>95%) attack at the oxirane carbon of *R* absolute configuration to give the *S*,*S* product. The stereoselectivity of isozyme C is very sensitive to the introduction and location of nitrogen substitution in the phenyl rings of phenanthrene 9,10-oxide. As a result isozyme C shows little or no stereoselectivity toward 4,5-diaza- and 4-azaphenanthrene 9,10-oxide. In contrast, 1-azaphenanthrene 9,10-oxide is attacked preferentially at the *R* carbon of the oxirane. The results suggest that hydrophobic interactions between the enzyme surface and the substrate distal to the oxirane ring are important in determining the stereoselectivity of the enzyme toward arene oxides.

Glutathione *S*-transferases (EC 2.5.1.18) catalyze the addition of the thiol of glutathione to electrophilic compounds with lipophilic substituents, a reaction of considerable importance in the detoxication of alkylating agents such as arene oxides, alkyl halides, and others (Chasseaud, 1979). Isozymes of GSH transferase[1] isolated from rat and human liver cytosol show a rather broad overlapping substrate specificity [for a recent review, see Jakoby & Habig (1980)]. Enzymes that catalyze biotransformation reactions of xenobiotics often exhibit a low degree of substrate selectivity, which is an obvious advantage for a catalyst required to act on structurally diverse and potentially toxic compounds. In contrast, the enzymatic processing of a xenobiotic compound can, and often does, proceed with a high degree of stereoselectivity in which stereochemical choices made by enzymes in the metabolic pathway affect the biological activity of the parent compound or its metabolites. For instance, cytochrome P-450 catalyzed oxidation at a prochiral center of an aromatic hydrocarbon can proceed stereoselectively to give predominantly one enantiomeric arene oxide (Levin et al., 1980; Armstrong et al., 1981a; van Bladeren et al., 1982). The initial stereoselection can influence the kinetic, stereochemical, and ultimate toxicological outcome of subsequent biotransformations as exemplified by the serial and stereoselective action of cytochrome P-450, epoxide hydrolase, and cytochrome P-450 on benzo[*a*]pyrene to give predominantly a single, highly tumorogenic stereoisomer of benzo[*a*]pyrene-7,8-diol 9,10-epoxide (Levin et al., 1980).

The efficacy of GSH transferases in the detoxication of xenobiotics may depend, in part, on the stereo- and enantioselectivity of the enzymes. Although van Bladeren et al. (1981) and Hernandez et al. (1980, 1981) have demonstrated GSH transferase to be stereoselective toward arene oxides and epoxides and similar findings have been reported for aralkyl halides (Mangold & Abdel-Monem, 1980) and phosphorothiolate triester substrates (Hutson, 1977), very little is known about the influence of substrate topology of GSH transferase catalyzed reactions. In a simple static sense, the active site of GSH transferase may be viewed as consisting of two subsites, one specific for glutathione and another relatively nonspecific lipophilic site for the electrophile. The chemical and structural features of the active site that dictate the substrate specificity and stereoselectivity of the enzyme are not known. In this paper we describe the use of arene and azaarene oxide substrates to probe the topology of the lipophilic surface at the active site of GSH transferase isozyme C from rat liver. The approach allows the enzyme to choose between two stereo- or regiochemically different reaction centers in a series of closely related substrates (Figure 1). From a comparison of the stereoselectivity of the enzyme toward phenanthrene 9,10-oxide and a series of azaphenanthrene 9,10-oxides, we conclude that hydrophobic interactions between the enzyme surface and the substrate distal to the oxirane ring are particularly important in determining the stereochemical outcome of the reaction.

Experimental Procedures

Materials

Enzymes. The three major isozymes, A, B, and C, of GSH transferase from rat liver were purified by combining the methods of Habig et al. (1974) and Guthenberg & Mannervik (1979) as follows. In a typical preparation, 100 g of liver was homogenized, extracted, chromatographed on DEAE-cellulose

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[1] Abbreviations: GSH transferase, glutathione *S*-transferase (EC 2.5.1.18); HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 4-morpholinepropanesulfonic acid; NH_4OAc, ammonium acetate; CD, circular dichroism; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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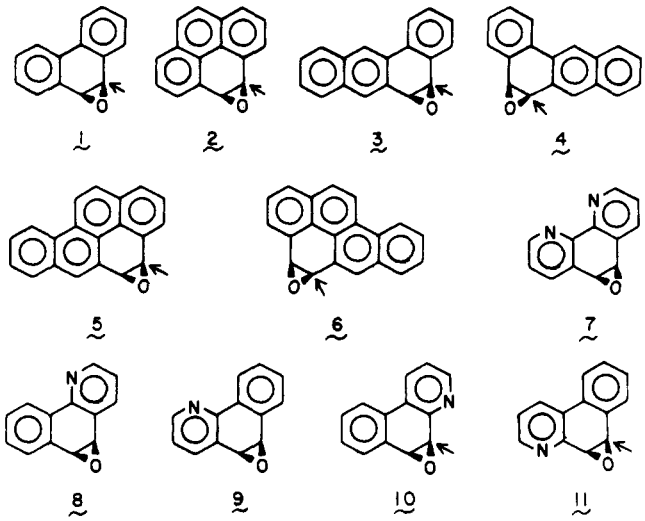

FIGURE 1: Structures of arene and azaarene oxide substrates. Oxirane rings are shown in the same orientation with the preferred site of attack of glutathione catalyzed by glutathione *S*-transferase isozyme C shown by arrows.

(Whatman DE-52), and precipitated with $(NH_4)_2SO_4$ as described by Habig et al. (1974). After dissolution in and dialysis against 10 mM Tris-HCl (pH 7.8), the solution was applied to and eluted from a 1.5 × 40 cm bed of *S*-hexylglutathionyl-Sepharose 6B as described by Guthenberg & Mannervik (1979). The mixture of isozymes was resolved by chromatography on a 1.5 × 40 cm bed of hydroxylapatite (Guthenberg & Mannervik, 1979). The three major fractions of the six peaks of protein and enzyme activity observed (1-chloro-2,4-dinitrobenzene as substrate) were concentrated and identified as isozymes B, C, and A (in order of elution) by substrate specificity (Habig & Jakoby, 1981) and discontinuous $NaDodSO_4$ gel electrophoresis (Scully & Mantle, 1981). Isozymes A, B, and C had specific activities of 4.8, 0.020, and 2.6 nmol min^{-1} mg^{-1}, respectively, with 1,2-dichloro-4-nitrobenzene as substrate. Isozymes A and C showed a single band of identical molecular weight on $NaDodSO_4$ gel electrophoresis. Isozyme B, as expected, showed two equally intense bands for the heterodimer and was contaminated with about 10% of an unidentified isozyme. UDPglucuronosyltransferase (EC 2.4.1.17) was purified to electrophoretic homogeneity with a modification (D. Lewis and R. N. Armstrong, unpublished results) of the method of Burchell & Weatherhill (1981).

Substrates. Phenanthrene 9,10-oxide, 4,5-diazaphenanthrene 9,10-oxide, racemic 1-azaphenanthrene 9,10-oxide, and racemic 4-azaphenanthrene 9,10-oxide were synthesized according to Krishnan et al. (1977). Pyrene 4,5-oxide was obtained by the method of Dansette & Jerina (1974). Enantiomers of benzo[*a*]pyrene 4,5-oxide were prepared as described by Chang et al. (1979) except that the diastereomeric *cis*-4,5-[(+)-α-methoxy-α-[(trifluoromethyl)phenyl]acetoxy]-4,5-dihydrobenzo[*a*]pyrenes were resolved by crystallization (hexane–CH_2Cl_2) of the 4*R*,5*S* isomer to diastereomeric purity (>99.7%), followed by preparative HPLC of the remaining mixture on a 21.2 × 250 mm Du Pont Zorbax SIL column eluted with hexane–CH_2Cl_2 (1:1) to give the 4*S*,5*R* isomer in >99.6% diastereomeric purity. Enantiomers of benz[*a*]anthracene 5,6-oxide and 9,10-dihydro-9,10-dihydroxyphenanthrene were synthesized as before (Armstrong et al., 1981b).

Products. Arene oxide (3–250 mM) in dioxane was added to an equal volume of an aqueous solution containing 1.2 equiv of glutathione and 3 equiv of NaOH under N_2. The mixture was stirred at room temperature until all the arene oxide had dissolved (3–18 h), extracted with 3 volumes of ether, adjusted to pH 4.5 with acetic acid, and lyophilized. Azaarene oxides were reacted in a similar fashion except the dioxane cosolvent was omitted. Positional isomers and diastereoisomers of the products were purified by reversed-phase HPLC on a 4.6 × 250 mm or 10 × 250 mm Altex ultrasphere ODS column eluted at 0.5 mL/min or 2.0 mL/min with the following solvent compositions for the glutathione conjugates: phenanthrene 9,10-oxide, 25 mM NH_4OAc (pH 4.0)–25% CH_3OH; pyrene 4,5-oxide, 75 mM NH_4OAc (pH 4.0)–30% CH_3OH; benz[*a*]anthracene 5,6-oxide, 25 mM NH_4OAc (pH 4.0)–40% CH_3OH; benzo[*a*]pyrene 4,5-oxide, 25 mM NH_4OAc (pH 4.0)–44% CH_3OH; 4,5-diazaphenanthrene 9,10-oxide, 0.2 M Tris–phosphate (pH 2.2)–5% CH_3OH; 1-azaphenanthrene 9,10-oxide and 4-azaphenanthrene 9,10-oxide, 75 mM NH_4OAc (pH 4.0)–15% CH_3OH. Amino acid analysis of the purified glutathione adducts gave the expected stoichiometric ratios of glutamate and glycine.

Monoglucuronides of (9*S*,10*S*)- and (9*R*,10*R*)-9,10-dihydro-9,10-dihydroxyphenanthrene were synthesized by reaction of the dihydrodiols (200 μM) with uridine 5′-diphosphoglucuronate (6 mM) in 30 mM Tris–12 mM $MgCl_2$ (pH 7.6) containing 2 mg/mL lysophosphatidylcholine in the presence of 1.4 units/mL rat liver UDPglucuronosyltransferase. Products were purified by HPLC on an Altex ultrasphere ODS column (10 × 250 mm) eluted with 0.1 M acetic acid–45% CH_3OH.

Other Materials. Glutathione, egg phosphatidylcholine, epoxy-activated Sepharose 6B and buffer salts were obtained from Sigma. Male Wistar rat livers were from Pel Freeze. Liposomes were prepared as described by Barenholz et al. (1977). *S*-Hexylglutathione (Vince et al., 1971) was coupled to epoxy-activated Sepharose 6B in 0.1 M carbonate (pH 10.5) at 40 °C for 50 h.

Methods

Kinetic Measurements. Initial rate measurements with water-soluble substrates, phenanthrene 9,10-oxide and 4,5-diazaphenanthrene 9,10-oxide, were measured spectrophotometrically at 290 nm ($\Delta\epsilon$ −3400 M^{-1} cm^{-1}) and 313 nm ($\Delta\epsilon$ 4000 M^{-1} cm^{-1}), respectively, in 50 mM Mops (pH 7.0) at 25 °C. Enzyme concentration was generally in the range of 0.02 and 0.2 μM (0.04–0.4 μM active sites). Substrate concentrations were varied between 15 and 200 μM at saturating (5 mM) glutathione. Reactions of lipophilic substrates catalyzed by GSH transferase isozyme C were measured spectrophotometrically in the presence of 2.0 μM unilamellar egg phosphatidylcholine vesicles (5.4 mM total phospholipid) in 50 mM Tris (pH 7.5) containing 5.0 mM glutathione at 25 °C. Wavelengths and $\Delta\epsilon$ (M^{-1} cm^{-1}) used for the substrates are as follows: phenanthrene 9,10-oxide, 290 nm, −4900; pyrene 4,5-oxide, 302 nm, −4100; (5*S*,6*R*)-benz[*a*]anthracene 5,6-oxide, 272 nm, −19 700; (5*R*,6*S*)-benz[*a*]anthracene 5,6-oxide, 272 nm, −25 700; (4*S*,5*R*)-benzo[*a*]pyrene 4,5-oxide, 276 nm, −15 600; (4*R*,5*S*)-benzo[*a*]pyrene 4,5-oxide, 276 nm, −23 400. Oxide concentrations were varied between 3.0 and 100 μM. All reactions were done in quadruplicate. Initial rate data were analyzed with the program HYPER (Cleland, 1979). The kinetic constant k_{cat} is the turnover number per active site.

Product Analysis. Reactions for product analysis were run to completion under the conditions described above for kinetic measurements except that substrate and enzyme concentrations were 50–100 μM and 1–5 μM, respectively. In all cases, the contribution of spontaneous reaction products was <2%.

Scheme I

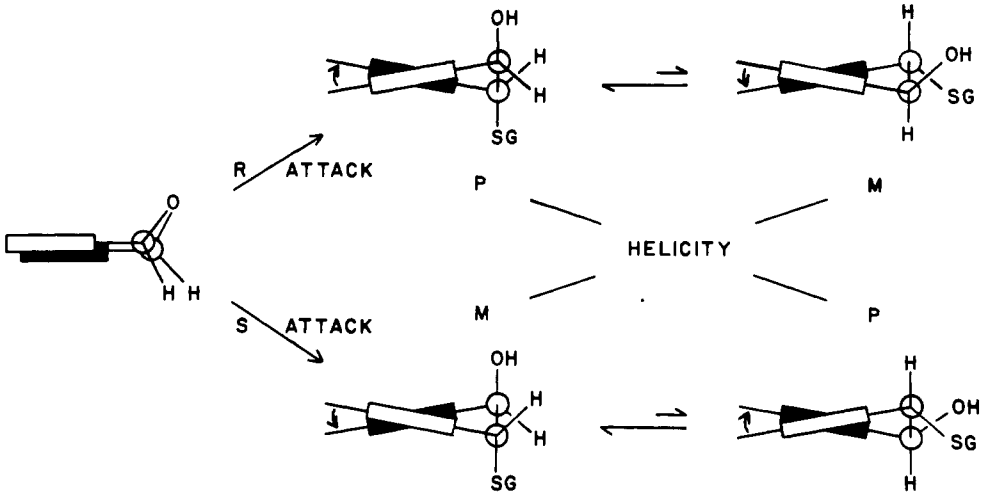

Table I: Kinetics and Stereoselectivity toward Phenanthrene 9,10-Oxide[a]

isozyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	% product isomer A
A	25 ± 1	45 ± 4	0.56	43
B	4.7 ± 0.5	140 ± 20	0.035	73
C	38 ± 1	31 ± 2	1.2	95

[a] Reactions were run at pH 7.0. Kinetics and products were analyzed as described under Methods.

Reaction mixtures (1.0 mL) were chromatographed on a 0.9 × 10 cm bed of Sephadex G-25 in 0.1 M NH_4OAc (pH 4.0). The products, which eluted between 1.1 and 6.0 column volumes, were lyophilized, redissolved in 0.1 mL NH_4OAc (pH 4.0), and analyzed by HPLC under the conditions described above. Ratios of the positional isomers or diastereoisomers were calculated on the basis of integrated peak areas at 254 nm. Values of ϵ_{254} were identical for diastereoisomers and within 10% of one another for positional isomers.

Instrumental Methods. Circular dichroism spectra were recorded at ambient temperature on either a JASCO J-41 or JASCO J-500C spectropolarimeter. Proton and broad-band proton-decoupled ^{13}C NMR spectra were obtained with an IBM/Bruker WP/200 or a Varian XL-100 spectrometer. ^1H and ^{13}C chemical shifts were determined relative to external tetramethylsilane.

Amino Acid Analysis. Glutathione conjugates were hydrolyzed in vacuo in 6 N HCl at 105 °C for 18 h for analysis on a Durrum analyzer.

Results

Kinetics and Stereoselectivity of Isozymes A, B, and C toward Phenanthrene 9,10-Oxide. Three major GSH transferases catalyze the addition of glutathione to phenanthrene 9,10-oxide with varying degrees of efficiency (Table I). Isozyme C is clearly the most efficient as judged by the turnover number, k_{cat}, and k_{cat}/K_m. Trans addition of glutathione to a nondissymmetric substrate such as phenanthrene 9,10-oxide (**1**) can occur by nucleophilic attack at either stereochemically distinct oxirane carbon to give two diastereomeric products (Scheme I). For instance, the specific base-catalyzed reaction gives equal amounts of the two diastereomers (**1A** and **1B**), which are separable by HPLC (Figure 2, inset). The enzyme-catalyzed reactions exhibit stereoselectivities that range from very low for isozyme A to very high for isozyme C (Table I).

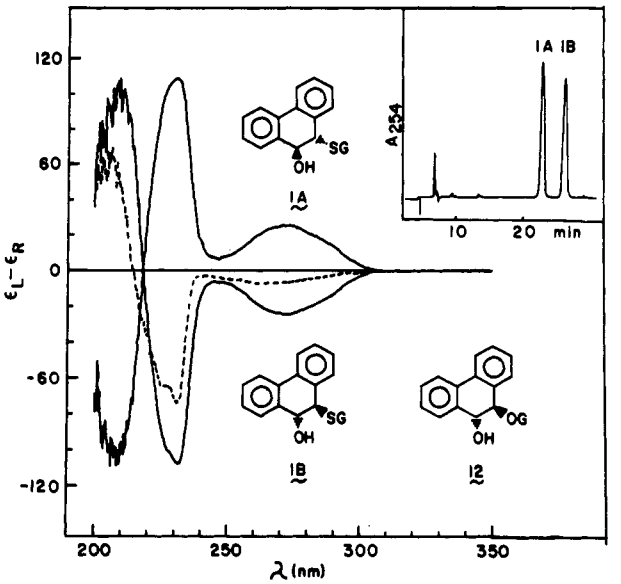

FIGURE 2: Separation and circular dichroism spectra of glutathione conjugates of phenanthrene 9,10-oxide. Separation of the 9*S*,10*S* (**1A**) and the 9*R*,10*R* (**1B**) diastereomers of 9-*S*-glutathionyl-10-hydroxy-9,10-dihydrophenanthrene (inset) was carried out as described under Methods. CD spectra of **1A**, **1B**, and the 9*R*,10*R* monoglucuronide, **12** (dashed line), were carried out in 25 mM NH_4OAc (pH 4.0). SG = glutathionyl; OG = glucuronosyl.

The absolute configuration of the diastereoisomers **1A** and **1B** (Figure 2) can be deduced from the chiroptical properties of the molecules, assuming the preferred solution conformation of the molecule is known. CD spectra of **1A** and **1B** (Figure 2) are mirror images of one another and quite similar to that of the *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene antipodes (Armstrong et al., 1981b), suggesting that the major contributor to the observed Cotton effects is the substituted 2,2′-bridged biphenyl chromophore with little contribution from the peptide. In principle, the twist sense or helicity about the axis defined by the biphenyl bond can be determined in 2,2′-bridged biphenyl systems such as **1A** and **1B** from the sign of the Cotton effect of the biphenyl conjugation band (Mislow & Hopps, 1962; Joshua et al., 1968). The helicity of the biphenyl axis is, of course, dependent on the thermodynamically preferred conformation of the molecule as illustrated in Scheme I.

The CD spectra of **1A** and **1B** show large ($\epsilon_L - \epsilon_R = \pm 23.9$) positive and negative Cotton effects at 273 nm, which from the following we believe to indicate the P and M helicities[2]

Table II: Kinetics and Stereoselectivity of Isozyme C[a]

substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	% isomer A[b]	configuration of isomer A
1, phenanthrene 9,10-oxide	25 ± 0.4	7.4 ± 0.6	3.4	96	9*S*,10*S*[c]
2, pyrene 4,5-oxide	4.7 ± 0.1	11 ± 1.2	0.43	96	4*S*,5*S*[d]
3, (−)-benz[*a*]anthracene 5(*R*),6(*S*)-oxide	16 ± 0.8	2.0 ± 0.5	8.0	99	5*S*,6*S*[e]
4, (+)-benz[*a*]anthracene 5(*S*),6(*R*)-oxide	5.0 ± 0.2	1.6 ± 0.2	3.1	98	5*S*,6*S*[e]
5, (−)-benzo[*a*]pyrene 4(*R*),5(*S*)-oxide	12 ± 0.6	4.6 ± 0.7	2.6	99	4*S*,5*S*[e]
6, (+)-benzo[*a*]pyrene 4(*S*),5(*R*)-oxide	4.1 ± 0.2	2.9 ± 0.5	1.4	99	4*S*,5*S*[e]

[a] Reactions were run at pH 7.5 in the presence of unilamellar phosphatidylcholine vesicles. [b] Isomer A refers to the more polar isomer eluting first on reversed-phase HPLC. [c] Absolute configuration of product determined as described in the text. [d] Absolute configuration deduced from close similarities between the CD spectra of the product (Figure 7) and those of **5A** and **6A** (Figure 9). [e] Absolute configurations previously determined (Armstrong et al., 1981a; van Bladeren et al., 1982).

of the chromophore, respectively. Due to the steric bulk of sulfur, it was expected that the favored conformation of **1A** and **1B** would be the one with diaxial substituents. NMR coupling constants ($J_{9,10}$) of the benzylic protons in the 9-glutathionyl-10-hydroxy-9,10-dihydrophenanthrene isomers **1A** [H(9), 4.62 ppm; H(10), 5.18 ppm; $J_{9,10}$ = 2.6 Hz] and **1B** [H(9), 4.60 ppm; H(10), 5.16 ppm; $J_{9,10}$ = 2.3 Hz] are small (<3 Hz), confirming this expectation. Comparison of the spectral properties of **1B** with those of (9*R*,10*R*)-9-(β-D-glucuronosyl)-10-hydroxy-9,10-dihydrophenanthrene (**12**) enzymatically synthesized from authentic (9*R*,10*R*)-dihydrodiol leads to the conclusion that **1B** has the M helicity. Proton NMR of the monoglucuronide [H(9), 4.28 ppm; H(10), 4.33 ppm; $J_{9,10}$ = 4.0 Hz] suggests the thermodynamically favored conformation places the hydroxyl and glucuronosyl substituents in the axial positions. This conformation and the known absolute configuration of the glucuronide then predicts the preferred helicity of the chromophore to be M. The CD spectra of the glucuronide are very similar to that of **1B** (Figure 2), having a large but somewhat lower circular dichroic extinction coefficient ($\epsilon_L - \epsilon_R = -6.77$) at 265 nm that is consistent with **1B** having the M helicity about the biphenyl axis.[3] It is concluded from the preferred conformation observed by NMR and the helicity of the chromophore observed by CD that isomers **1A** and **1B** have the 9*S*,10*S* and 9*R*,10*R* absolute configurations, respectively.

Kinetics and Stereoselectivity of Isozyme C toward K-Region Arene Oxides. Isozyme C catalyzes the stereoselective attack of glutathione on the oxirane carbon of *R* absolute configuration in phenanthrene 9,10-oxide as shown above. The sensitivity of the kinetics and stereoselection of isozyme C to alterations in substrate structure in a series of K-region arene oxides (Figure 1) is shown in Table II. It should be noted that the reactions require the addition of detergent or phospholipid vesicles to solubilize the more lipophilic substrates, **2**–**6**. Since the K_m values will depend to some extent on the partitioning of substrates between the vesicle phase and aqueous phase, strict comparison between different substrates is difficult (Lu et al., 1977; Armstrong et al., 1980). Two things are readily apparent from the data of Table II. First, the enzyme under these conditions shows little kinetic discrimination between substrates in the series in that all of the kinetic constants are within 1 order of magnitude of one another. Second, in all cases the enzyme catalyzes attack predominantly at the *R* carbon of the oxirane to yield the product of *S*,*S* configuration. The nondissymmetric substrate, pyrene 4,5-oxide, gives predominantly the diastereomeric product **2A**, which is concluded to have the 4*S*,5*S* absolute configuration on the basis of the positive CD transition at 270 nm (Figure 7; see paragraph at end of paper regarding supplementary material) as compared to the positive transitions of **5A** and **6A** of known configuration (Armstrong et al., 1981a). A consequence of the enzyme-catalyzed attack at the *R* carbon is that the enzyme is observed to change its regioselectivity toward the antipodes of the chiral substrates, **3** and **4** and **5** and **6**. That is, the predominant product from **3** is the 5-glutathionyl isomer **3A** and that from **4** is the 6-glutathionyl isomer **4A** (see Figure 8 structures). Similarly, the action of isozyme C on enantiomers **5** and **6** yields predominantly the 4-glutathionyl and 5-glutathionyl isomers **5A** and **6A**, respectively (Figure 9).

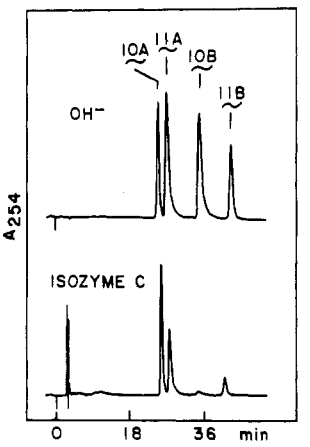

FIGURE 3: HPLC separation of glutathione conjugates of racemic 1-azaphenanthrene 9,10-oxide. Separations were done on a 4.6 × 250 mm Altex ultrasphere ODS column eluted at 0.5 mL/min with 75 mM NH_4OAc (pH 4.0) and 15% CH_3OH. Upper trace shows the specific base-catalyzed reaction. Lower trace shows the predominance of isomer **10A** and **11A** from the isozyme C catalyzed reaction.

[2] We have chosen to specify the asymmetry of the biphenyl chromophore in terms of conformational chirality or the helical sense of the biphenyl axis rather than in terms of axial chirality (Cahn et al., 1966). Thus, in the biphenyl series the M and P helicities are equivalent to the *R* and *S* axial chiralities, respectively. One advantage of specifying the helical sense of the biphenyl axis is that the designation M or P is insensitive to the atomic substitution on the axis as in the case of the 2,3′-bridged phenyl-2′-pyridyl chromophore of **8A**, **8B**, **9A**, and **9B**.

[3] It should be noted that the sign of Cotton effects in the optical rotary dispersion curves of a series of substituted 2,2′-bridged biphenyls at the long-wavelength transition is also consistent with this assignment (Mislow et al., 1962). It is important to point out that the solvent can dramatically influence the conformer population and hence the chiroptical properties of the glucuronide and of the related *trans*-dihydrodiol (D. Lewis, D. Cobb, and R. N. Armstrong, unpublished results). In organic solvents (CH_3OH or acetone), the *trans*-dihydrodiol has been correctly predicted from NMR studies of related compounds (Jerina et al., 1976; Bruice et al., 1976) to prefer the conformation with diequatorial substituents. However, in water, the conformer population shifts to favor that with the hydroxyl groups diaxial. Thus, in water, the (9*S*,10*S*)-dihydrodiol has a CD spectrum (Armstrong et al., 1981b) consistent with a biphenyl chromophore of P helicity.

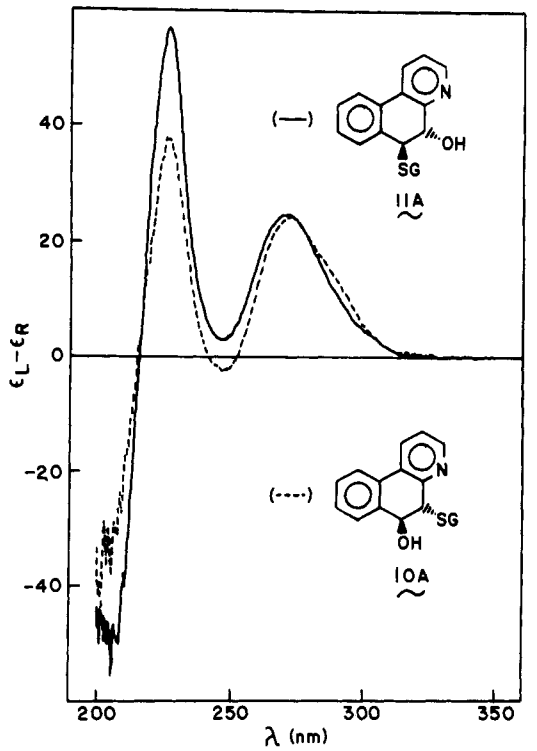

FIGURE 4: CD spectra of isomers **10A** and **11A**. Spectra were obtained in 25 mM NH_4OAc (pH 4.0). Concentration of the isomers used to calculate $\epsilon_L - \epsilon_R$ was obtained by using $\epsilon_{272} = 12\,700\ M^{-1}\ cm^{-1}$ at pH 4.0.

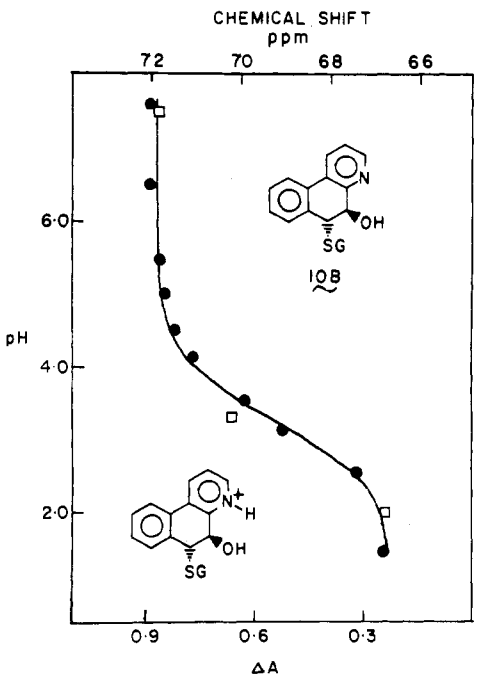

FIGURE 5: Spectrophotometric and ^{13}C NMR titration of **10B**. Spectrophotometric titration (●) was carried out with 40 μM **10B** by following ($\Delta A = A_{244} - A_{318}$) the difference in between the isosbestic point at 244 nm and the pyridinium ion absorption band at 318 nm. The solid line is a computer fit of the data with pK_a of 3.27 ± 0.02. ^{13}C chemical shifts of the carbinol carbon (□) were obtained from a sample of **10B** (10 mg/mL in D_2O) (~20 000 pulses, 0.573-s acquisition time) and are reported relative to external tetramethylsilane standard.

Glutathione Conjugates of Azaarene Oxides. Introduction of electronegative heteroatoms into the hydrophobic phenyl rings of phenanthrene 9,10-oxide as in the azaarene oxides **7–11** yields substrates of much higher hydrophilicity with little change in steric bulk. Specific base-catalyzed reaction of glutathione with the nondissymetric 4,5-diazaphenanthrene 9,10-oxide (**7**) gives the two expected diastereomeric products, which are separable by HPLC. CD spectra (Figure 10) of the products **7A** and **7B** are mirror images of one another and quite similar to those of **1A** and **1B** (Figure 2). Diastereomers **7A** and **7B** are assigned the 9*S*,10*S* and 9*R*,10*R* absolute configurations, respectively, through comparison of the sign of the CD transitions between 320 and 200 nm with those of **1A** and **1B**.

Reaction of racemic 1-azaphenanthrene 9,10-oxide (**10** + **11**) with glutathione gives two diastereomeric pairs of positional isomers that are resolved by HPLC as shown in Figure 3. Identification of the isomers was achieved by examination of their chiroptical and ^{13}C magnetic resonance properties. The two different positional isomers can be distinguished (though not identified) by distinct differences in their UV (data not shown) and CD spectra (Figures 4 and 12). Furthermore, pairing of the two different positional isomers from a given enantiomer can be accomplished from their CD spectra (Figure 12) as follows. Antiparallel attack of glutathione on the oxirane carbon of *R* or *S* absolute configuration will yield the 9*S*,10*S* or 9*R*,10*R* stereoisomers, respectively, having the opposite preferred axial helicity of the chromophore. As a result, the two positional isomers from the *same* enantiomer should have CD transitions of *opposite* sign. This is, in fact, observed in the glutathione conjugates of **3–6** (Figures 8 and 9), where the absolute configuration of the isomers are known (Armstrong et al., 1981a; van Bladeren et al., 1982). Isomers **10A** and **11A** (Figure 3), therefore, are different positional isomers from the two enantiomeric oxides and are assigned the 9*S*,10*S* absolute configurations on the basis of the close resemblance of their CD spectra to that of **1A** (compare Figures 2 and 4). If one of the positional isomers can be identified, then, from correlation of the CD spectra, the structures of the other three isomers can be deduced.

At which position the glutathionyl moiety is attached can be determined by the relative sensitivity of the ^{13}C chemical shifts of the hydroxyl- and thioether-bearing carbons to the protonation state of the pyridine ring. The relatively small (0.6-ppm) chemical shift difference between the benzylic (56.9 ppm) and pyridylic carbons (57.5 ppm) ($CHCl_3$) of racemic 1-azaphenanthrene 9,10-oxide is expected to increase to ~20 ppm for the difference between hydroxyl- (~70 ppm) and thioether- (~50 ppm) bearing carbons (Hylarides et al., 1979; Cox et al., 1981) in the glutathione adducts of **10** and **11**. More importantly, the chemical shift of the pyridylic carbon, C(10), should be much more sensitive to the protonation state of the pyridine ring than the benzylic carbon, C(9). The pK_a of the pyridinium ion of **10B**, the only isomer that was isolable in large quantity, is 3.27 ± 0.02 as determined by spectrophotometric titration shown in Figure 5. The hydroxyl- and thioether-bearing carbons of **10B** exhibit resonances at 71.90 and 48.69 ppm at pH* 7.6 that are shifted upfield by 5.10 and 1.09 ppm at pH* 2.0, respectively. Furthermore, the upfield shift seems to be associated with the pK_a of the pyridinium ion (Figure 5). The magnitude and direction of the chemical-shift change is consistent with that observed in the model compound 2-pyridylcarbinol in which the carbinol carbon resonance shifts 4.1 ppm upfield from 64.9 ppm at pH* 7.6 to 60.8 ppm at pH* 0.35 in D_2O. On the basis of the magnitude, direction, and pH dependence of the chemical shift change, it is concluded that the carbinol carbon is proximal to the pyridine ring in **10B**. The combined NMR and CD data suggest that **10B** is (9*R*,10*R*)-1-aza-9-*S*-glutathionyl-10-

hydroxy-9,10-dihydrophenanthrene. From this assignment and the CD spectra of the other isomers, the structures of **10A**, **11A**, and **11B** can be deduced (Figure 12). It should be noted that the two 9-OH positional isomers **10A** and **11B** are particularly unstable at pH ≤ 4 ($t_{1/2} \sim 12$ h at 25 °C), presumably due to the increased acidity of the proton at the pyridylic carbon C(10), leading to the trans elimination of H_2O.

Reaction of racemic 4-azaphenanthrene 9,10-oxide (**8** + **9**) afforded the expected four isomeric conjugates that elute on HPLC [4.6 × 250 mm ultrasphere ODS, 75 mM NH_4OAc (pH 4.0)–15% CH_3OH, 0.5 mL/min) as follows: **8A** (24.5 min), **8B** (33.3 min), **9A** (21.5 min), and **9B** (36.3 min). Following the same reasoning as above, we used the CD spectra of the four isomers (Figure 11) to pair the different positional isomers from the same enantiomeric oxide. Comparison of the CD spectra of **9A** (Figure 11) to that of **1A** (Figure 1) suggests **9A** to have the 9*S*,10*S* absolute configuration. The pK_a of the pyridinium ion of **9A** is 3.56 ± 0.02 as determined by spectrophotometric titration (data not shown). The chemical shift of the carbinol carbon in **9A** shifts 1.69 ppm upfield from 70.99 ppm (pH* 7.1) to 69.30 ppm (pH* 1.2) while the thioether-bearing carbon shifts 0.55 ppm upfield from 48.40 (pH* 7.1) to 47.85 ppm (pH* 1.2) upon protonation of the pyridine ring. Similarly, the carbinol carbon resonance in the appropriate model compound, 3-pyridylcarbinol, shifts 0.94 ppm upfield from 62.05 ppm (pH* 9.6) to 61.11 ppm (pH* 0.67). On the basis of the larger sensitivity of the carbinol carbon in **9A** to protonation of the pyridine ring, it is concluded that the hydroxyl group in **9A** is at the C(10) position and that **9A** is (9*S*,10*S*)-4-aza-9-*S*-glutathionyl-10-hydroxy-9,10-dihydrophenanthrene. Structures of the other three isomers (Figure 11) follow from correlation of their CD spectra with that of **9A** (Figure 11). As expected, the chemical shift of the thioether-bearing carbon of the other positional isomer, **9B**, which shifts 1.03 ppm upfield from 48.76 ppm (pH* 7.6) to 47.73 ppm (pH 2.0), is more sensitive to protonation of the pyridine ring than the carbinol carbon, which shifts 0.61 ppm upfield (71.16–70.65 ppm) in the same pH range.

Kinetics and Stereoselectivity of Isozyme C toward Azaarene Oxides. The azaarene oxides **7–11** are good substrates for GSH transferase isozyme C. For instance, kinetic constants for **7** with isozyme C (K_m = 110 ± 8 μM, k_{cat} = 76 ± 3 min^{-1}, k_{cat}/K_m = 0.69 μM^{-1} min^{-1}) are comparable to those for **1** (Table I). The stereoselectivity of the enzyme is remarkably sensitive to the introduction and location of nitrogen substitution in the phenyl ring of phenanthrene 9,10-oxide (Table III). The enzyme shows little or no stereoselectivity toward **7–9**, where either the 4 or 4 and 5 positions are substituted with nitrogen. Interestingly, the stereoselectivity is only slightly affected by heteroatom substitution in the 1 position adjacent to the oxirane ring as in **10** and **11**. Thus the structural information required for stereorecognition of **1** by isozyme C appears to reside, in part, in a portion of the substrate molecule distal to the oxirane ring.

Discussion

Assignment of Absolute Configurations by CD Spectroscopy. The assignment of absolute configuration to the glutathione conjugates by circular dichroism spectroscopy must be viewed with some caution since it is a comparative technique. However, it is observed in the glutathione conjugates of benz[*a*]anthracene 5,6-oxide (**3A–4B**), benzo[*a*]pyrene 4,5-oxide (**5A–6B**), and monoglucuronide, **12**, all of known absolute configuration, that the isomers of *S*,*S* and *R*,*R* configurations exhibit positive and negative circular dichroic extinction coefficients, respectively, for their major (most intense) long-wavelength CD transitions (240–280 nm). In addition, all of the molecules almost certainly have the same preferred solution conformation (Scheme I). In all cases, pairs of diastereomers give essentially mirror-image CD spectra, indicating that the chirality of the peptide has little or no influence on the CD transitions observed from 200–350 nm. On this basis, it is reasonable to assign absolute configurations from the sign of the major CD transition between 240 and 280 nm. In the series of bridged biphenyl, phenylpyridyl, and bipyridyl compounds, which have strikingly similar CD spectra from 200–350 nm (Figures 2, 4, and 10–12), there should be little doubt of the assignment of molecular chirality by this technique.

Table III: Stereoselectivity of Isozyme C with Azaarene Oxides[a]

substrate	% isomer A	absolute configuration of isomer A[c]
7, 4,5-diazaphenanthrene 9,10-oxide	55	9*S*,10*S*
8, (9*S*,10*R*)-4-azaphenanthrene 9,10-oxide[b]	53	9*R*,10*R*
9, (9*R*,10*S*)-4-azaphenanthrene 9,10-oxide[b]	57	9*S*,10*S*
10, (9*S*,10*R*)-1-azaphenanthrene 9,10-oxide[b]	92	9*S*,10*S*
11, (9*R*,10*S*)-1-azaphenanthrene 9,10-oxide[b]	79	9*S*,10*S*

[a] Reactions were run to completion in 50 mM Mops, pH 7.0, and analyzed by HPLC as described under Methods. [b] Enantiomers **8** and **9** and **10** and **11** were run as their racemic mixtures. [c] Absolute configurations are assigned as described in the text.

Stereoselectivity of GSH Transferase. The broad substrate specificity of many enzymes involved in the metabolism of xenobiotics suggests that catalysis by these enzymes demands much less in terms of mutual structural interaction between the substrate and the enzyme surface than do enzymes evolved to catalyze specific transformations. Although GSH transferase is very specific for glutathione, it seems to require little structural information from the electrophile other than a hydrophobic surface (Keen et al., 1976). Determining the shape of the hydrophobic surface is one key to understanding the substrate and stereoselectivity of the enzyme. K-region arene oxides are particularly well-suited probes of the active site of GSH transferase in that the enzyme is free to choose between the two isomeric transition states for reaction at the two stereo- or regiochemically distinct oxirane carbons. One particular geometric requirement is that productive substrate binding must occur with the oxirane ring situated for trans-antiparallel attack of the sulfur nucleophile. Therefore, in principle, the topology of the active site relative to the thiolate nucleophile of glutathione can be deduced.

For any discussion of the molecular basis of stereoselection by GSH transferase some knowledge of the active site homogeneity in the dimeric protein must be assumed. For instance, the marked loss in stereoselectivity of isozyme C between phenanthrene 9,10-oxide and 4,5-diazaphenanthrene 9,10-oxide can be interpreted differently depending on the catalytic efficacy and subunit composition of the isozyme. Three possibilities exist. First, the enzyme can consist of two catalytically identical subunits. Second, the enzyme can be a heterodimer in which only one subunit effectively turns over both substrates. Finally, the enzyme can be a heterodimer in which both subunits are capable of turning over one or both of the substrates. In the first two cases the change in ste-

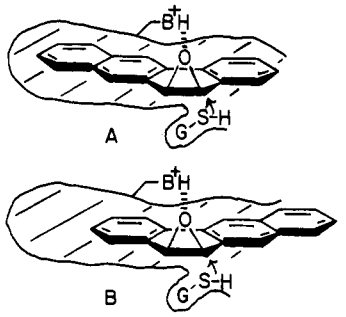

FIGURE 6: Illustration of possible asymmetries in the active site of the C subunit of GSH transferase isozyme C. Diagram A shows productive binding of (5*R*,6*S*)-benz[*a*]anthracene 5,6-oxide with glutathione (GSH) attack at the 5 position. In (B), maximum hydrophobic overlap between enzyme and substrate and/or efficient protonation of the leaving group dictates glutathione attack at the six position of the 5*S*,6*R* enantiomer.

reoselectivity toward **1** and **7** can be attributed to alterations in the mutual interaction of the substrates and a single active site while in the latter case the loss of stereoselectivity can be due to "mixed" catalysis with the substrates and two structurally different active sites.

After submission of the manuscript a report (Mannervik & Jensson, 1982) appeared showing isozyme A to be a homodimer with a subunit composition designated A_2 and isozyme C to be a heterodimer with subunit composition AC. An isozyme with subunit composition C_2 was also reported. The stereoselectivity of isozymes A and C toward phenanthrene 9,10-oxide clearly indicates that this substrate is processed primarily (≥90%) through the C subunit of the heterodimeric isozyme C. Furthermore, given the kinetic constants (Table I), the subunit composition of isozymes A and C, the conditions of the stereoselectivity experiments (substrate concentration $\leq K_m$), and the assumption of no product inhibition, it can be estimated that a minimum of 75% of **1** is processed through the C subunit of isozyme C. Isozyme A has been found to be considerably less efficient than isozyme C toward **2–6**, a fact that has precluded the accurate determination of kinetic constants for these substrates but that further suggests that **2–6** are acted on primarily by the C subunit of heterodimeric isozyme C.[4]

Given that the arene and azaarene oxide substrates are processed primarily through the C subunit of isozyme C, it is interesting to speculate on the origins of the stereoselectivity of the subunit. Two mechanisms exist, though they are not mutually exclusive. First, the primary determinant in the stereoselection may be recognition of the local chirality of the oxirane carbon being attacked, for instance, through asymmetric positioning of a critical general acid (required for protonation of the leaving group) with respect to the approach of the sulfur nucleophile. Alternatively, the stereoselectivity may be the result of asymmetry in the hydrophobic surface of the substrate binding site. Both possibilities are illustrated in Figure 6 and are viable explanations for the change in regioselectivity of the enzyme with the antipodes **3** and **4** and **5** and **6**. In fact, the stereoselectivity of isozyme C as illustrated in Figure 1 is consistent with the enzyme orienting the substrate for maximum hydrophobic overlap between the substrate and an asymmetric enzyme surface as proposed in Figure 6.

Phenanthrene 9,10-oxide clearly contains the structural features necessary for stereochemical recognition by isozyme C. The 95 to 5 ratio of attack at the carbons of *R* and *S* absolute configuration in **1** suggests a difference of about 1.7 kcal/mol in the free energies of activation for the two diastereomeric transition states. If hydrophobic interactions are important in determining the stereoselectivity of isozyme C, then the small free energy difference between the two transition states may be perturbed by introduction of hydrophilic substituents in the substrate. Thus, one interpretation of the loss of stereoselectivity toward phenanthrene 9,10-oxide upon introduction of nitrogen in the 4 and 5 positions of the molecule as in **7–9** is that the polar heteroatom interrupts the hydrophobic interaction critical to the stereoselection. Placing the heteroatom adjacent to the oxirane as in **10** and **11** has a much smaller effect on the observed stereoselectivity, where again the oxirane carbon of *R* absolute configuration is preferentially attacked in both substrates. The resulting change in regioselectivity between **10** and **11** argues that electronic differences between the two oxirane carbons have little influence on the observed stereoselectivity. These results suggest that hydrophobic interactions between the substrate and enzyme surface distal to the oxirane ring are important in determining the stereoselectivity of isozyme C.

Efficacy in Detoxication of Xenobiotics. The ability of the glutathione *S*-transferases to participate in the detoxication of a given xenobiotic or metabolite is a complex function of the relative amounts and catalytic efficacy of the several isozymes found in vivo. For instance, isozymes A and C are relatively good catalysts for the model arene oxide, **1** (Table I), while isozyme B is clearly inferior toward this substrate. The ranking of catalytic efficiency toward **1** roughly parallels that reported for racemic benzo[*a*]pyrene 4,5-oxide (Jakoby et al., 1976). Interestingly, the first report of the stereoselectivity of GSH transferases toward arene oxides (Hernandez et al., 1980) that employed whole rat liver cytosol and racemic benzo[*a*]pyrene 4,5-oxide concluded that two regioisomeric products, subsequently identified as **5A** and **6A** (Armstrong et al., 1981a), predominate. This would suggest that this substrate is processed primarily through isozyme C or another isozyme with similar stereoselectivity in vivo. Unlike epoxide hydrolase (Armstrong et al., 1981b), GSH transferase C shows little kinetic discrimination between chiral substrates **3–6** in the presence of phosphatidylcholine vesicles. Thus prior stereoselection by the cytochromes P-450 in the 4,5- and 5,6-oxidations of benzo[*a*]pyrene and benz[*a*]anthracene may not directly limit the efficiency of conjugation of these arene oxides with glutathione. Whether this is true with other arene oxides remains to be seen.

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Supplementary Material Available

Six figures (Figures 7–12) providing circular dichroism spectra and structures of the glutathione conjugates of **2–11** (6 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 585-08-0; **1A**, 84107-68-6; **1B**, 84107-69-7; **2**, 37496-00-7; **2A**, 84066-23-9; **2B**, 84142-52-9; **3**, 74444-65-8; **3A**, 82534-96-1; **3B**, 82484-09-1; **4**, 74444-64-7; **4A**, 82534-97-2; **4B**,

[4] Preliminary evidence with the homodimeric isozyme having the C_2 subunit composition shows that this isozyme catalyzes preferential attack (≥95%) at the *R* carbon of phenanthrene 9,10-oxide but shows a much lower degree of stereoselectivity (~75% *R* attack) with 4,5-diazaphenanthrene 9,10-oxide (C. Boehlert and R. N. Armstrong, unpublished results). Thus, the stereoselectivity of isozyme C seems to parallel that of the homodimeric C_2 isozyme.

82534-98-3; **5**, 72010-12-9; **5A**, 78478-20-3; **5B**, 78478-21-4; **6**, 72010-13-0; **6A**, 78478-19-0; **6B**, 78479-11-5; **7**, 65115-91-5; **7A**, 84066-24-0; **7B**, 84107-70-0; **8**, 84107-64-2; **8A**, 84107-72-2; **8B**, 84066-25-1; **9**, 84107-65-3; **9A**, 84066-26-2; **9B**, 84107-71-1; **10**, 84107-66-4; **10A**, 84066-27-3; **10B**, 84107-74-4; **11**, 84107-67-5; **11A**, 84066-28-4; **11B**, 84107-73-3; **12**, 84066-22-8; glutathione *S*-transferase, 50812-37-8.

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