Stereoselectivity and Regioselectivity of Uridine-5′-diphosphoglucuronosyltransferase toward Vicinal Dihydrodiols of Polycyclic Aromatic Hydrocarbons†

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ABSTRACT: The ability of a purified rat liver microsomal uridine-5′-diphosphoglucuronosyltransferase to catalyze the glucuronidation of stereoisomeric *trans*- and *cis*-9,10-dihydroxy-9,10-dihydrophenanthrenes and 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes is examined. The enzyme shows the ability to discriminate kinetically between the antipodes of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene with turnover numbers of 0.070 and 1.4 s^{-1} and k_c/K_{mapp} values of 4.4×10^3 and 1.1×10^3 M^{-1} s^{-1} for the 9*R*,10*R* and 9*S*,10*S* stereoisomers. Glucuronidation of the nondissymmetric *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene proceeds with a turnover number of 0.037 s^{-1} and k_c/K_{mapp} of 18×10^3 M^{-1} s^{-1} to give a 60/40 mixture of the two possible diastereomeric products. Three of the four stereoisomers of 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene are regioselectively glucuronidated by the enzyme with a high degree of kinetic discrimination. Turnover numbers for the 4*S*,5*S*, 4*R*,5*R*, and 4*S*,5*R* stereoisomers are 4.1, 0.37, and 0.23 s^{-1} with k_c/K_{mapp} values of 23.8×10^3, 0.23×10^3, and 3.15×10^3 M^{-1} s^{-1}, respectively. The 4*R*,5*S* cis isomer is not a substrate. Enzyme-catalyzed reactions of the 4*S*,5*S* and 4*S*,5*R* isomers give exclusively (≥95%) the 4-glucuronide with the 4*R*,5*R* isomer giving the 5-glucuronide. The kinetic and regiochemical results indicate that the enzyme recognizes hydroxyl groups on the β-face or bottom face of the 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes. Thus the reaction is more dependent on the facial specificity of the enzyme than on the relative configuration of the vicinal hydroxyl groups. The stereochemical results obtained here and the stereoselectivities of cytochrome P-450$_c$ and epoxide hydrolase predict that if glucuronidation can participate effectively in the metabolism of benzo[*a*]pyrene 4,5-dihydrodiols, the major isomeric glucuronide formed will be (4*R*,5*R*)-5-β-D-glucuronosyl-4-hydroxy-4,5-dihydrobenzo[*a*]pyrene.

Microsomal UDPglucuronosyltransferases[1] (EC 2.4.1.17) are important enzymes involved in the detoxication of both endogenous and xenobiotic compounds [for a recent review, see Kasper & Henton (1980)]. The enzymes catalyze the transfer of the glucuronosyl group of UDP-glucuronate to a variety of structurally diverse nucleophiles. Though much of the broad substrate selectivity of the microsomal enzyme system is attributed to the presence of multiple enzyme forms (Burchell, 1980; Weatherill & Burchell, 1980; Matern et al., 1982; Owens & Mackenzie, 1982), enzyme preparations that are apparently homogeneous also exhibit a rather low degree of substrate selectivity (Magdalow et al., 1982; Matern et al., 1982), a property not uncommon to enzymes catalyzing detoxication reactions. Methods that probe the factors controlling the substrate selectivity of the enzymes are of considerable value in understanding the role of glucuronidation in the metabolism of xenobiotics.

Vicinal dihydrodiols are important intermediates in the mammalian and microbial metabolism of polycyclic aromatic hydrocarbons. Although conflicting reports have appeared concerning the ability of the rat liver microsomal enzyme(s) to catalyze the glucuronidation of vicinal diols (Nemoto & Gelboin, 1976; Nemoto, 1978; Fahl et al., 1978; Bansal et al., 1981), it is clear from the present work that vicinal dihydrodiols of polycyclic aromatic hydrocarbons are, in fact, substrates for the purified enzyme. The ability of UDP-glucuronosyltransferase to participate in the metabolism of dihydrodiols can depend in part on the stereoselectivity of the enzyme since the stereoselectivity and regioselectivity of the prior enzymes in the metabolic pathway, the cytochromes P-450 and epoxide hydrolase, often lead to predominant formation of the *R*,*R* antipodes of dihydrodiol metabolites (Huberman et al., 1976; Thakker et al., 1977, 1979; Nordquist et al., 1981). Although stereoselective glucuronidation of several drugs has been demonstrated [for example, see Thompson et al. (1981)], nothing is known about the stereo- and regioselectivity of UDPglucuronosyltransferase toward vicinal dihydrodiol substrates. In a more general sense, these substrates are potentially useful probes of the topology of the active site of UDPglucuronosyltransferase in that they provide stereo- or regiochemically distinct reaction centers between which the enzyme may choose.

In this paper, the ability of a purified rat liver UDPglucuronosyltransferase to discriminate between stereo- and regiochemically distinct hydroxyl groups of the *cis*- and *trans*-9,10-dihydroxy-9,10-dihydrophenanthrenes and 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes (see Figure 1 for structures) is examined. The results show that the enzyme can discriminate between the three stereoisomers of 9,10-dihydroxy-9,10-dihydrophenanthrene (**1**–**3**). In addition, the enzyme exhibits a large kinetic and regiochemical discrimination toward the four stereoisomers of 4,5-dihydroxy-4,5-

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[1] Abbreviations: UDPglucuronosyltransferases, uridine-5′-diphosphoglucuronosyltransferase; UDPGA, uridine 5′-diphosphoglucuronic acid; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DSS, 3-(trimethylsilyl)-1-propanesulfonate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

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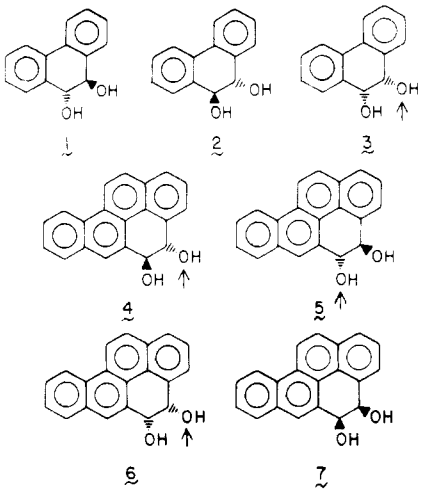


FIGURE 1: Structures of dihydrodiol substrates. Arrows indicate preferred sites of enzyme-catalyzed glucuronidation.

dihydrobenzo[*a*]pyrene (**4**–**7**). The stereo- and regioselectivity of the enzyme-catalyzed reaction suggests that only hydroxyl groups on the β-face[2] of 4,5-dihydroxy-4,5-dihydrobenzo[*a*]-pyrene are recognized by the enzyme.

Experimental Procedures

Materials

General Materials. Uridine 5′-diphosphoglucuronate, dithiothreitol, Lubrol PX, egg lysophosphatidylcholine, and *Escherichia coli* β-glucuronidase were obtained from Sigma. Uridine-5′-diphosphohexanolamine was prepared and coupled to CNBr-activated Sepharose 4B as described by Barker et al. (1972). Male Wistar rat livers were from Pel-Freeze, Inc. All other reagents and buffer salts were of the highest quality commercially available.

Substrates. The 9*R*,10*R*, **1**, and 9*S*,10*S*, **2**, antipodes of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene were resolved as before (Armstrong et al., 1981a). *cis*-9,10-Dihydroxy-9,10-dihydrophenanthrene, **3**, was obtained from the hydrocarbon (Dansette & Jerina, 1974). Racemic *trans*-4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene was prepared from the cis isomer (Platt & Oesch, 1982). The 4*S*,5*S*, **4**, and 4*R*,5*R*, **5**, trans enantiomers were resolved as described by Kedzierski et al. (1981). The 4*S*,5*R* and 4*R*,5*S* antipodes **6** and **7** of *cis*-4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene were obtained as previously described (Chang et al., 1979; Cobb et al., 1983a). 4-Hydroxybenzo[*a*]pyrene, **8**, and 5-hydroxybenzo-[*a*]pyrene, **9**, were prepared by acid-catalyzed dehydration of the *cis*-dihydrodiol as described for 5- and 6-hydroxybenz-[*a*]anthracene (Wiley et al., 1975).

Enzyme. UDPglucuronosyltransferase was purified by the following modification of the method of Burchell & Weatherill (1981). In a typical preparation, 60 g of chopped rat liver suspended in 250 mL of 50 mM Tris–0.15 M KCl (pH 7.5) was homogenized by 2 30-s bursts in a Waring blender followed by 10 strokes in a Teflon-coated glass homogenizer. Cell debris was removed by centrifugation for 20 min at 13000*g*. Microsomes were harvested from the supernatant by centrifugation (1 h at 105000*g*), washed with 10 mM EDTA–0.15 M KCl (pH 7.4), repelleted at 105000*g*, and stored at −80 °C in 25 mM Tris–0.1 mM DTT (pH 8.0) (buffer A) containing 10% glycerol. Microsomes (75 mL, 20 mg of protein/mL) were solubilized by adjusting the solution to contain 0.8% Lubrol PX, followed by stirring for 0.5 h at 4 °C. The solution was diluted to 300 mL with buffer A, 10% in glycerol, and centrifuged 2 h at 105000*g*. The supernatant was placed on a DEAE-cellulose column (Whatman DE-52, 2.5 × 38 cm) equilibrated with buffer A containing 0.05% Lubrol PX and 20% glycerol. After this was washed with 250 mL of buffer, the enzyme was eluted with a 500-mL linear gradient of 0–0.3 M KCl in the equilibration buffer. The sharp peak of enzyme activity that eluted at the leading edge of the major protein peak was collected and dialyzed against 4 L of buffer B [25 mM Tris, 0.5 mM DTT, 0.05% Lubrol PX, 20% glycerol (pH 8.0)]. After dialysis, solid $MgCl_2$ was added to a final concentration of 5 mM, and the solution was applied to a 2.5 × 5.5 cm bed of UDP-hexanolamine–Sepharose 4B equilibrated with buffer B containing 5 mM $MgCl_2$. The column was washed with buffer B, and the enzyme was eluted with a 6-mL aliquot of 5 mM UDPGA in buffer B. The enzyme was concentrated by adsorption on a 0.5 × 1 cm bed of DEAE-cellulose in buffer B followed by elution with 0.3 M KCl in buffer B. UDPGA could be removed by chromatography on Sephadex G-25 in buffer B. The enzyme was flash frozen and stored at −80 °C in buffer B.

Methods

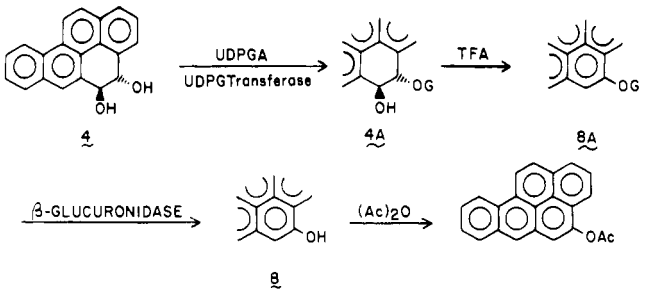
Standard Assay. Enzyme activity was measured at 25 °C in 40 mM Tris, 16 mM $MgCl_2$, and 7.8 mM UDPGA (pH 7.5) containing 1 mg/mL lysophosphatidylcholine with a continuous spectrophotometric assay that used *p*-nitrophenol (0.6 mM) as the acceptor substrate by following the decrease in absorbance at 440 nm ($\Delta\epsilon = -3300\ M^{-1}\ cm^{-1}$).

Kinetics of Glucuronidation of Dihydrodiols. Glucuronidation of dihydrodiols was monitored by a HPLC assay as follows. Reactions were run in 33 mM Tris, 13.2 mM $MgCl_2$, and 2.0 mg/mL lysophosphatidylcholine (pH 7.5) containing 7.8 mM UDPGA at 25 °C. Dihydrodiols were added in dioxane solution, and reactions were initiated by addition of enzyme to a final concentration of 0.1–0.5 μM. Final volume of reaction mixtures was 50 μL containing 4% v/v dioxane. After incubation for 5–60 min, reactions were quenched by addition of 50 μL of CH_3OH at 0 °C. A 20-μL aliquot of the quenched reaction was analyzed by HPLC on a IBM 9533 chromatograph equipped with a Kratos 773 variable-wavelength detector, a Hewlett-Packard 3390A recording integrator, and an Altex Ultrasphere ODS column (4.6 × 250 mm). All reactions were run in triplicate, maintaining substrate conversions in the range of 2–10%. Data were analyzed by using the computer program HYPER (Cleland, 1979). Turnover numbers were calculated by assuming one active site per 55 000 daltons.

For the kinetic studies, the optimal chromatographic conditions for separation of the substrate and product from other reactants and their retention times at a flow rate of 0.5 mL/min are as follows: **1**, 45% CH_3OH in 0.1 M acetic acid, (**1**) 39 min, (**1A**) 16 min; **2**, 50% CH_3OH in 0.1 M acetic acid, (**2**) 27 min, (**2A**) 18 min; **3**, 45% CH_3OH in 0.1 M acetic acid, (**3**) 39 min, (**3A** and **3B**) 23 min; **4**, 74% CH_3OH in 0.1 M acetic acid, (**4**) 18 min, (**4A**) 12 min; **5**, 60% CH_3OH in 0.1 M acetic acid for 10 min then 60–80% CH_3OH at 2%/min, (**5**) 22 min, (**5A**) 12 min; **6**, 50% CH_3OH for 10 min then 50–75% CH_3OH at 1.67%/min, (**6**) 31 min, (**6A**) 27 min.

[2] The designation of the stereotopic faces of benzo[*a*]pyrene is derived from the rules suggested by Rose et al. (1980). Thus, facial nomenclature used here is based on the handedness (clockwise rotation) of the atomic numbering convention for the molecule such that the top face of the molecule is designated α. The reader should be aware that this designation is opposite that previously used by others to describe the relative stereochemistry of the diastereomeric 7,8-diol 9,10-epoxides of benzo[*a*]pyrene [see Buening et al. (1978) for structures] by analogy to the facial nomenclature for steroids.

Scheme I



Isolation of Products. In general, preparative reactions were carried out as follows. Reactions (1–10 mL) were run under conditions identical with those used in kinetic studies except that substrate and enzyme concentrations were 0.2–2 mM and 0.4–3 μM, respectively. Reactions were monitored by HPLC as described in the text. Products from **1–3** were purified by chromatography on Sephadex LH-20 (2.5 × 100 cm) in 0.1 M acetic acid where the glucuronides **1A**, **2A**, **3A**, and **3B** had normalized elution volumes, V_e/V_0, of 3.0, 3.8, 5.5, and 4.7, respectively. Glucuronides from **4–6** were purified by preparative HPLC on an Altex Ultrasphere ODS column (1.0 × 25 cm) eluted with 68% CH_3OH in 0.1 M acetic acid. Solvents were removed by lyophilization.

Regiochemical Analysis of Products. The position of glucuronidation of **4–6** was determined as indicated in Scheme I. Between 20 and 300 nmol of glucuronide was dehydrated by dissolution in 50 μL of anhydrous TFA for 15 min at room temperature. After removal of TFA with a stream of dry N_2, the phenol glucuronides **8A** and **9A** were dissolved in CH_3OH, and a portion was analyzed by HPLC. The remainder was dissolved in 0.25 mL of 50 mM KH_2PO_4 (pH 6.8) containing 2000 IU of β-glucuronidase (*p*-nitrophenyl β-glucuronide as substrate) for 10 min and extracted with 2 volumes of ethyl acetate. The extract was dried over Na_2SO_4. After removal of the solvent with N_2, the sample was acetylated with acetic anhydride in pyridine (80 °C, 10 min). Acetoxybenzo[*a*]-pyrenes were identified by gas–liquid chromatography as previously described (Armstrong et al., 1981b).

Instrumental Methods. Circular dichroism spectra were recorded on a JASCO J-500C spectropolarimeter with signal averaging of 8–32 spectra with a DP-500N data processor; NMR spectra were obtained in ^2H_2O at 200 MHz on an IBM WP200 SY spectrometer. Chemical shifts are reported relative to external DSS.

Results

Properties of UDPglucuronosyltransferase. The enzyme can be prepared routinely in 2–8% yield by the procedure described with a reproducible specific activity of 1000 ± 100 nmol min^{-1} mg^{-1} when assayed with *p*-nitrophenol. The enzyme is stable indefinitely at −80 °C. The protein was characterized by an $E_{280}^{0.1\%}$ = 2.2 [protein concentration based on the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard] and exhibited a single band on $NaDodSO_4$ gel electrophoresis with an apparent molecular weight of 55 000. UDPglucuronosyltransferase purified here has properties similar to the *p*-nitrophenol conjugating enzymes previously isolated from rat liver microsomes (Gorski & Kasper, 1977; Burchell & Weatherill, 1981).

Glucuronidation of 9,10-Dihydroxy-9,10-dihydrophenanthrenes. Racemic *trans*-9,10-dihydrodroxy-9,10-dihydrophenanthrene (**1** + **2**) is smoothly glucuronidated by UDPglucuronosyltransferase in a preparative-scale reaction to yield two diastereomeric products that are separable by HPLC as illustrated in Figure 2. The two products **1A** and **2A** arise from monoglucuronidation of the 9*R*,10*R* and 9*S*,10*S* antipodes **1** and **2**, respectively. That **1A** and **2A** are, in fact, monoglucuronides is clear from their integrated ^1H NMR spectra in which the ratio of anomeric protons [**1A**, H(1′), 4.43 ppm, $J_{1',2'}$ = 7.8 Hz; **2A**, H(1′), 4.65 ppm, $J_{1',2'}$ = 8.0 Hz] to benzylic protons [**1A**, H(9), 5.02 ppm, and H(10), 4.97 ppm, $J_{9,10}$ = 3.9 Hz; **2A**, H(9) and H(10), 4.98 ppm, $J_{9,10}$ = 3.5 Hz][3] is 1 to 2. No evidence for diglucuronidation of **1** of **2** was observed even at extended reaction times of several days.

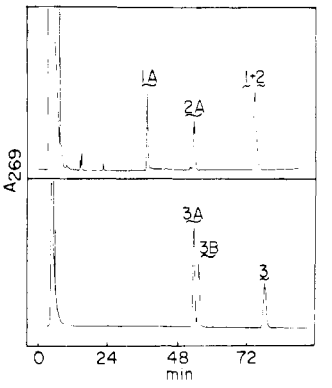


FIGURE 2: HPLC separation of stereoisomeric glucuronides of racemic *trans*-, **1** + **2** (upper trace), and *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene, **3** (lower trace), in enzyme-catalyzed reaction mixtures. Reactions were run to approximately 50% completion and chromatographed on a Rainin Microsorb C18 column (4.6 × 250 mm) eluted at 0.5 mL/min with a gradient (0.33%/min) of 30–40% CH_3OH in 0.1 M acetic acid.

The nondissymmetric *cis*-dihydrodiol, **3**, has chemically identical but stereochemically distinct hydroxyl groups such that monoglucuronidation can give two diastereomeric products. Enzyme-catalyzed glucuronidation of **3** gave a 60/40 mixture of the two diastereomers **3A** and **3B**, which were separable by HPLC (Figure 2). The products had the expected 1 to 2 ratio of anomeric [**3A**, H(1′), 4.74 ppm, $J_{1',2'}$ = 7.6 Hz; **3B**, H(1′), 4.51 ppm; $J_{1',2'}$ = 7.4 Hz] to benzylic protons [**3A**, H(9), 5.06 ppm, and H(10), 4.99 ppm, $J_{9,10}$ = 3.5 Hz; **3B**, H(9), 5.07 ppm, and H(10), 5.03 ppm, $J_{9,10}$ = 3.5 Hz]. Again no evidence for formation of the diglucuronide was found.

Assignment of absolute configuration to **3A** and **3B** is not a trivial problem. However, a tentative assignment is made here on the basis of a comparison of the chiroptical properties of the *cis*-glucuronides to those described for the *trans*-glucuronides (Cobb et al., 1983b). Circular dichroism and ^1H NMR studies have demonstrated that the conformer populations of **1A** and **2A** are very sensitive to solvent composition (Cobb et al., 1983b). For instance, $\Delta\epsilon_{228}$ for **1A** indicates that the mole fraction of conformer with the glucuronosyl and hydroxyl groups diaxial decreases from 0.86 in H_2O to 0.38 in CH_3OH. A similar decrease is calculated for **2A**. The CD spectra of **3A** and **3B** (Figure 3) also show solvent-dependent shifts in conformer populations. The circular dichroic extinction coefficient $\Delta\epsilon_{228}$, the magnitude and sign of which measures the average helicity of the biphenyl chromophore, becomes more negative (increase in M helicity) for **3A** and more positive (increase in P helicity) for **3B** as the solvent is changed from H_2O to CH_3OH. If it is assumed that the

[3] The fortuitous identity of the chemical shifts of the benzylic protons of **2A** precludes a direct determination of $J_{9,10}$ since the two protons are magnetically equivalent. However, $J_{9,10}$ can be obtained from the natural abundance ^{13}C satellite proton resonances 74 Hz downfield of the singlet at 4.98 ppm.

Table I: Kinetics of Glucuronidation of 9,10-Dihydroxy-9,10-dihydrophenanthrenes[a]

substrate	absolute configuration	K_{mapp} (μM)	k_c (s^{-1})	k_c/K_{mapp} ($\times 10^3$ M^{-1} s^{-1})
1	9*R*,10*R*	16.1 ± 0.8	0.070 ± 0.001	4.4
2	9*S*,10*S*	1280 ± 70	1.40 ± 0.03	1.1
3	9*R*,10*S*	2.1 ± 0.6[b]	0.037 ± 0.002	18

[a] Reactions were run as described under Methods. [b] The relative large error (~30%) in K_m arises from the fact that the K_m is below the sensitivity limits of the HPLC assay in which the lowest substrate concentration used was 7 μM.

glucuronosyl group will show a greater preference for the axial position in H_2O than in CH_3OH, then the solvent-dependent change in helicities of the biphenyl chromophores indicates that **3A** and **3B** have the 9*S*,10*R* and 9*R*,10*S* absolute configurations, respectively. On the basis of this analysis, the enzyme shows a slight preference for catalyzing reaction at the carbinol group of *S* absolute configuration.

Kinetics of Reaction with 9,10-Dihydroxy-9,10-dihydrophenanthrenes. Kinetic constants for the enzyme-catalyzed reactions of **1–3** at a single, high concentration of UDPGA are summarized in Table I. Turnover numbers for the three substrates show **2** to be turned over 20–40 times more rapidly than **1** or **3**. In contrast, the magnitudes of k_c/K_{mapp} show the opposite trend and are largely determined by sizable differences in K_{mapp} for the three substrates.

Glucuronidation of 4,5-Dihydroxy-4,5-dihydrobenzo[a]pyrenes. Three (**4–6**) of the four stereoisomeric 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes are substrates for UDPglucuronosyltransferase. Although two regioisomeric products are possible from each substrate, only a single product from each of the three substrates is evident from HPLC analysis of reaction mixtures (data not shown). In addition, the three products **4A–6A** obtained from the enzyme-catalyzed glucuronidation of **4–6**, respectively, can be separated from one another by HPLC as illustrated in Figure 4. The 4-, and 5-hydroxybenzo[*a*]pyrenyl glucuronides **8A** and **9A** are also separable under the same chromatographic conditions (Figure 4), a fact taken advantage of in determining the regiochemistry of the enzyme-catalyzed reaction below. The regiochemical purity and identity of the three products can be established by the chemical strategy illustrated in Scheme I for the **4** to **4A** transformation. Dehydration of both **4A** and **6A** with anhydrous trifluoroacetic acid gave exclusively (≥95%) 4-hydroxybenzo[*a*]pyrenyl glucuronide, **8A**, as judged by cochromatography (Figure 4) with **8A** prepared by enzyme-catalyzed glucuronidation of 4-hydroxybenzo[*a*]pyrene, **8**. In contrast, dehydration of **5A** gave 5-hydroxybenzo[*a*]pyrenyl glucuronide, **9A**, in >90% regioisomeric purity. Further degradation of each of the dehydration products with β-glucuronidase followed by acetylation resulted in the expected acetoxybenzo[*a*]pyrenes in high (≥95%) regioisomeric purity as determined by gas–liquid chromatography (Armstrong et al., 1981b). These results, summarized in Table II, clearly show that UDPglucuronosyltransferase exhibits a high regioselectivity toward three of the four stereoisomeric 4,5-dihydrodiols of benzo[*a*]pyrene. The (4*R*,5*S*)-*cis*-dihydrodiol, **7**, is not glucuronidated by the purified enzyme under the conditions employed.

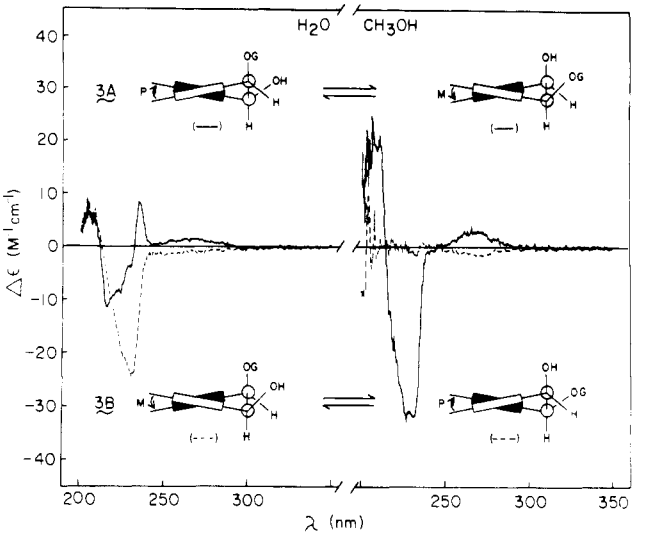


FIGURE 3: Circular dichroism spectra of glucuronides **3A** and **3B** in CH_3OH and H_2O. Circular dichroic extinction coefficients, $\Delta\epsilon_{228}$, for **3A** changes from −3.78 M^{-1} cm^{-1} in H_2O to −31.4 M^{-1} cm^{-1} in CH_3OH. For **3B**, $\Delta\epsilon_{228}$ is −21.1 M^{-1} cm^{-1} in H_2O and −0.65 M^{-1} cm^{-1} in CH_3OH. If one assumes the glucuronosyl group will show a greater preference for the axial position in H_2O than in CH_3OH (Cobb et al., 1983b), the mole fraction of conformer with the glucuronosyl group axial changes from 0.48 to 0.34 for **3A** as the solvent is changed from H_2O to CH_3OH. Similarly, conformer populations of 0.61 and 0.50 are calculated for **3B** in H_2O and CH_3OH, respectively.

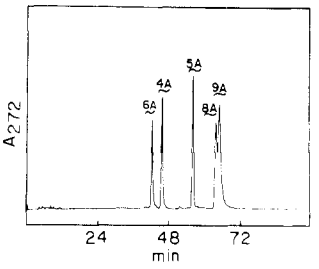


FIGURE 4: HPLC separation of products obtained from enzyme-catalyzed glucuronidation of the stereoisomers of 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene. Products isolated from reactions of individual substrates were combined and chromatographed on a Rainin Microsorb C18 column (4.6 × 250 mm) eluted at 0.5 mL/min with a gradient (0.5%/min) of 50–65% CH_3OH in 0.1 M acetic acid.

Kinetics of Glucuronidation of 4,5-Dihydroxy-4,5-dihydrobenzo[a]pyrenes. Kinetic constants for the glucuronidation of **4–6** at a high concentration of UDPGA are found in Table II. The enzyme shows a kinetic preference for the substrates in the order of **4 > 6 > 5 >> 7** when the bimolecular rate constant k_c/K_{mapp} is used as the criterion of catalytic efficiency. Combining this information with the regioselectivity of the enzyme and the known absolute configurations of the substrates, it is clear that the enzyme catalyzes glucuronidation of hydroxyl groups that are on the β-face of the molecule (see Figure 1). The two best substrates, **4** and **6**, have 4β-hydroxyl groups that are glucuronidated. The

Table II: Kinetics and Regioselectivity of UDPglucuronosyltransferase toward 4,5-Dihydroxy-4,5-dihydrobenzo[*a*]pyrene

substrate	absolute configuration	position of glucuronidation	k_c (s^{-1})	K_{mapp} (μM)	k_c/K_{mapp} ($\times 10^3$ M^{-1} s^{-1})
4	4*S*,5*S*	4	4.1 ± 0.7	172 ± 47	23.8
5	4*R*,5*R*	5	0.37 ± 0.15	1620 ± 450	0.23
6	4*S*,5*R*	4	0.23 ± 0.02	73 ± 15	3.15
7	4*R*,5*S*				<0.05

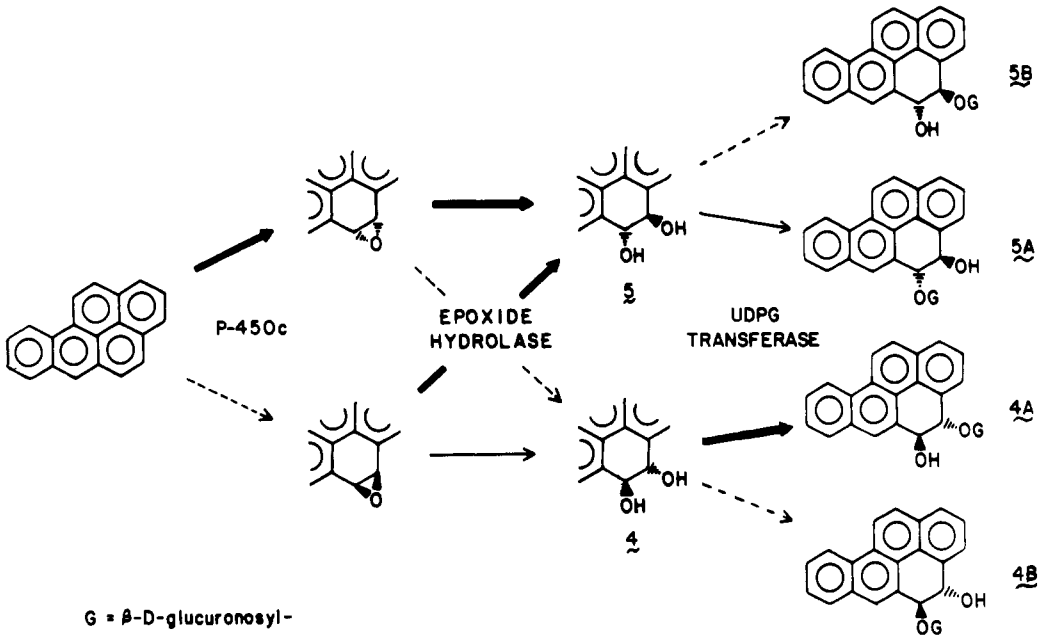


FIGURE 5: Stereochemical map of metabolism of benzo[*a*]pyrene at the 4,5-position derived from stereoselective behavior of purified rat liver microsomal cytochrome P-450$_c$, epoxide hydrolase, and UDPglucuronosyltransferase. Heavy, light, and dashed arrows indicate major, minor, and unobserved pathways, respectively.

efficiency of the enzyme toward **5**, which has a 5β-hydroxyl group, is diminished by a relatively large K_{mapp}. In the case where both hydroxyl groups are on the α-face as in **7**, no reaction is observed.

Discussion

Enzyme-Catalyzed Glucuronidation of Vicinal Diols. Purified UDPglucuronosyltransferase appears quite competent in catalyzing the glucuronidation of both *trans*- and *cis*-dihydrodiols of polycyclic aromatic hydrocarbons in micellar solutions of lysophosphatidylcholine. Turnover numbers for the substrates examined here are in the range of 0.05–4 s^{-1} and are comparable to turnover numbers for other enzymes involved in metabolism of polycyclic aromatic hydrocarbons. For instance, epoxide hydrolase and glutathione *S*-transferase have turnover numbers on the order of 1 s^{-1} for phenanthrene 9,10-oxide (Armstrong et al., 1980; Cobb et al., 1983a). From a metabolic standpoint, it is perhaps more appropriate to evaluate the catalytic efficiency of the enzyme with the bimolecular rate constant k_c/K_{mapp} since it seems unlikely that dihydrodiol metabolites would ever accumulate to concentrations in excess of K_{mapp}. Again, the magnitude of k_c/K_{mapp} for UDPglucuronosyltransferase ($\sim 10^4$ M^{-1} s^{-1}) is comparable to those for both epoxide hydrolase and glutathione *S*-transferase.

There exist, however, substantial difficulties in extrapolation of kinetic data in micellar solutions to an in vivo situation for a membrane-bound enzyme, particularly an enzyme such as UDPglucuronosyltransferase which exhibits catalytic properties dependent on phospholipid–protein interactions (Gorski & Kasper, 1978; Hochman et al., 1981; Magdalou et al., 1982; Singh et al., 1982). At the very least, micellar solutions provide a membrane mimetic environment into which hydrophobic substrates may partition (Armstrong et al., 1980, 1981a). Although it is clear that much of the substrate[4] and perhaps the enzyme is micelle bound, it is not clear whether the micelle-associated or free dihydrodiol is the actual substrate for the enzyme. The profound influence of detergents, phospholipids, and microsomal membrane environments on the catalytic activity and substrate selectivity of the enzyme (Zakim & Vessey, 1976; Magdalou et al., 1982) may be responsible for the conflicting reports (Nemoto & Gelboin, 1976; Bansal et al., 1981) concerning the ability of the microsomal enzyme to catalyze the glucuronidation of dihydrodiols. Indeed, attempts to isolate large amounts of glucuronides of dihydrodiols from reactions catalyzed by microsomes for stereochemical analysis have not yet been successful.[5] This remains an area of active investigation.

Stereoselectivity of UDPglucuronosyltransferase. The enzyme-catalyzed reactions with the stereoisomers of 9,10-dihydroxy-9,10-dihydrophenanthrenes show some interesting kinetic differences that deserve comment. Although the bimolecular rate constants for the glucuronidation of **1**–**3** are of the same order of magnitude, rather substantial differences in k_c and K_{mapp} are apparent for the three substrates. The relative values of k_c and K_{mapp} for **1** and **3** suggest that these two stereoisomers may undergo a substantial amount of nonproductive binding. The 9*S*,10*S* stereoisomer, **2**, has by far the largest k_c and K_{mapp}, perhaps suggesting that the enzyme is able to utilize the intrinsic binding energy (Jencks, 1975) of **2** for catalysis more effectively than that of **1** and **3** where it is manifest in the ground state, resulting in much lower values of K_{mapp}. A more detailed examination of the kinetic behavior of the reactions is required to address this point effectively.

The cis isomer, **3**, provides a simple example of a nondissymmetric substrate with two chemically identical but stereochemically distinct carbinol groups. In this instance, the enzyme is barely able to discriminate between the two reaction centers, giving a 60/40 mixture of the two diastereomeric products. The active site of the enzyme must then be able to accommodate two productive binding modes for this small achiral substrate.

The kinetics and regiochemistry of glucuronidation of the 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes by UDPgluc-

[4] In the presence of aqueous lysophosphatidylcholine (2 mg/mL), **2** (25 μM) has circular dichroic extinction coefficient $\Delta\epsilon_{228} = -5.33$, suggesting that the average solvent environment of **2** in the micellar solution is substantially less polar than H_2O (Cobb et al., 1983b).

[5] D. A. Lewis and R. N. Armstrong, unpublished results.

uronosyltransferase (Figure 1, Table II) indicates that the enzyme is highly regioselective for a given stereoisomer and is sensitive to both the absolute and relative configurations of the carbinol groups. Perhaps the most interesting stereochemical generalization that is evident from the reactions of **4**–**7** is that the enzyme appears to recognize only hydroxyl groups on the β-face of the 4,5-dihydrobenzo[*a*]pyrenes (see Figure 1). In the instance where both hydroxyl groups are on the β-face of the substrate (i.e., **6**), the enzyme clearly prefers reaction at the 4-position. In addition to the facial specificity toward **4**–**7**, the enzyme shows clear kinetic preferences for carbinol groups of different regiochemical identity and relative configuration. The order of kinetic preference appears to be trans-4β > cis-4β > trans-5β > cis-5β.

The facial specificity of UDPglucuronosyltransferase indicates that the topology of the active site is such that the benzo ring of the substrate can be accommodated in only a relatively restricted set of orientations. Otherwise, it should be possible for **4**, **5**, or **7** to bind "upside down" in productive complexes where the hydroxyl groups on the α-face of the molecule would react. A more detailed picture of the aglycon binding site will have to await the study of a wider range of dihydrodiol substrates. Finally, it should be pointed out that dihydrodiol substrates can exist in two conformers with the hydroxyl groups diaxial or diequitorial in the trans isomers or axial or equatorial in the cis isomers. Therefore, it is ultimately also of interest to ascertain the conformational preference of productive enzyme–substrate complexes.

Glucuronidation and Metabolism of Benzo[a]pyrene at the 4,5-Position. The ability of UDPglucuronosyltransferase to participate in the mammalian metabolism of benzo[*a*]pyrene at the 4,5-position and the identity of any resulting glucuronides depend on the stereoselectivities of all the enzymes in the metabolic pathway. As an example, a metabolic map constructed from the known stereoselectivities of purified rat liver microsomal cytochrome P-450$_c$ (Armstrong et al., 1981b), epoxide hydrolse (Armstrong et al., 1981a), and UDPglucuronosyltransferase is illustrated in Figure 5. Of course, metabolic maps constructed with purified enzymes need not accurately reflect the situation in vivo since participation of other isozymes or the consequences of a variety of other possible metabolic events are not considered. However, if one bears in mind this limitation, some interesting observations concerning the involvement of glucuronidation in the metabolism of benzo[*a*]pyrene at the 4,5-position can be made. First, the initial steps in the metabolic pathway are compatible in a stereochemical sense. That is, the (4*S*,5*R*)-benzo[*a*]pyrene 4,5-oxide generated by cytochrome P-450$_c$ catalyzed oxidation of the hydrocarbon is the better of the two possible enantiomeric substrates for epoxide hydrolase. Second, the stereoselectivity of UDPglucuronosyltransferase is not kinetically compatible with that of the previous steps in that the dihydrodiol (**5**) produced is a poor substrate for the enzyme. Finally, if glucuronidation does take place, only one, **5A**, of the four possible isomeric products would be expected from the in vivo processing of benzo[*a*]pyrene at the 4,5-position of the hydrocarbon. An assessment of the stereoselective compatibility of UDPglucuronosyltransferase with other routes of metabolism of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons is under active investigation.

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Registry No. **1**, 64440-29-5; **1A**, 84066-22-8; **2**, 23190-41-2; **2A**, 87206-15-3; **3**, 2510-71-6; **3A**, 87861-12-9; **3B**, 87861-13-0; **4**, 64474-18-6; **4A**, 87830-60-2; **5**, 62600-10-6; **5A**, 87830-61-3; **6**, 72040-29-0; **6A**, 87861-14-1; **7**, 72040-30-3; **8A**, 60262-84-2; **9A**, 87830-62-4; EC 2.4.1.17, 9030-08-4.

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Photoaffinity Labeling of the Sodium- and Potassium-Activated Adenosinetriphosphatase with a Cardiac Glycoside Containing the Photoactive Group on the C-17 Side Chain†

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ABSTRACT: The synthesis and properties of a radiolabeled glycoside photoaffinity probe, [^{3}H]-(3β,5β,14β,20*E*)-24-azido-3-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-21-norchol-20(22)-en-23-one, containing the photoactive group at the C-17 side chain of the steroid moiety are reported. The molecule binds to the sodium- and potassium-activated adenosinetriphosphatase from porcine kidney outer medulla under type II binding conditions [5 mM $MgCl_2$, 3 mM phosphate, 2 mM ethylenediaminetetraacetic acid, 30 mM tris(hydroxymethyl)aminomethane, pH 7.2, 37 °C] in the dark with an equilibrium dissociation constant of (1.4 ± 0.3) × 10^{-7} M. Ultraviolet irradiation of a solution of enzyme plus ^{3}H-labeled probe, followed by analysis of covalently incorporated radiolabel, shows ouabain-displaceable labeling exclusively of the α subunit of the sodium- and potassium-activated adenosinetriphosphatase. These data indicate that the binding site of the C-17 side group of cardiac glycosides is located on or near the α subunit of this enzyme.

Digitalis glycosides are widely used in the treatment of cardiac dysrhythmias and congestive heart failure (Fullerton, 1982). The physiological receptor for these drugs is thought to be Na,K-ATPase[1] (Schwartz et al., 1982; Akera, 1977).

While the precise quaternary structure of the Na,K-ATPase catalytic complex is not yet known (Kyte, 1981), the protein is thought to be composed of α (M_r 100000) and β (M_r 40000) subunits (Peterson & Hokin, 1981; Freytag & Reynolds, 1981; Peters et al., 1981) and perhaps a low molecular weight proteolipid component (M_r 12000) (Forbush et al., 1978; Rodgers & Lazdunski, 1979a; Collins et al., 1982). The α subunit of the enzyme contains the site of phosphorylation by ATP (Post & Kume, 1973; Nishigaki et al., 1974); however, the exact location of the cardiac glycoside binding site remains somewhat unclear. Forbush et al. (1978), using photoaffinity labels of ouabain with the photoactive group attached to the sugar portion of the molecule, found covalent labeling of the α subunit as well as a low molecular weight proteolipid component. Rodgers & Lazdunski (1979a,b) showed that if the photoactive group was located on the sugar group of ouabain (NAP-ouabain), labeling occurred at both the α subunit and the low molecular weight peptide, while experiments utilizing a glycoside derivative with the group positioned between the steroid A and B rings (NAP-strophanthidin) gave covalent label incorporation only in the α subunit. In experiments probing the sugar binding sites of digitoxin, Hall & Ruoho (1980) synthesized 3‴- and 4‴-(diazomalonyl)digitoxin and found that both α and β subunits were covalently labeled, with enhanced labeling of the β subunit for the 4‴-derivative as opposed to the 3‴-derivative. At this time, there has been no report of successful photoaffinity-labeling or affinity-alkylating studies of the cardiac glycoside binding site where the alkylating moiety is located at or near the C-17 side group of the glycoside.

Previous work (Fullerton et al., 1979; Ahmed et al., 1983) has indicated that a strong correlation exists between the C-17 side group carbonyl oxygen position and the resulting inhibition of Na,K-ATPase activity. The purpose of this paper is to report the synthesis and binding properties of a radiolabeled cardiac glycoside photoaffinity probe with the photoactive group located on the C-17 side chain of the molecule. Photoaffinity-labeling experiments, followed by analysis for co-

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[1] Abbreviations: Na,K-ATPase, magnesium-dependent, sodium- and potassium-stimulated adenosinetriphosphatase (EC 3.6.1.3); ATP, adenosine 5′-triphosphate; NAP-strophanthidin, *N*-strophanthidinyl-*N*′-(2-nitro-4-azidophenyl)ethylenediamine; NAP-ouabain, *N*-ouabainyl-*N*′-(2-nitro-4-azidophenyl)ethylenediamine; 24-azidodigitoxoside (**1**), (3β,5β,14β,20*E*)-24-azido-3-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl)-oxy]-14-hydroxy-21-norchol-20(22)-en-23-one; digitoxigenin digitoxoside acetonide, (3β,5β)-3-[[2,6-dideoxy-3,4-*O*-(1-methylethylidene)-β-D-*ribo*-hexopyranosyl]oxy]-14-hydroxycard-20(22)-enolide; TLC, thin-layer chromatography on silica gel; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; THF, tetrahydrofuran; DMF, dimethylformamide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; solvent system A, CH_2Cl_2–ethyl acetate–methanol (20:2:1); solvent system B, CH_2Cl_2–ethyl acetate–methanol (10:3:1); solvent system F, CH_2Cl_2–acetone (1:1); EDTA, ethylenediaminetetraacetic acid.

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