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Glutathione Conjugation of Aflatoxin B_1 *exo*- and *endo*-Epoxides by Rat and Human Glutathione *S*-Transferases

Kevin D. Raney,† David J. Meyer,‡ Brian Ketterer,‡ Thomas M. Harris,† and F. Peter Guengerich*,§

Departments of Chemistry and Biochemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, Tennessee 37232-0146, and Cancer Research Campaign Molecular Toxicology Group, University College and Middlesex School of Medicine, London W1P 6DB, U.K.

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Much evidence supports the view that the rate of conjugation of glutathione (GSH) with aflatoxin B_1 (AFB_1) *exo*-epoxide is an important factor in determining the species variation in risk to aflatoxins and that induction of GSH *S*-transferases can yield a significant protective effect. An assay has been developed in which the enzymatic formation of the conjugates of GSH and AFB_1 *exo*-epoxide and the recently described AFB_1 *endo*-epoxide is measured directly. ^1H NMR spectra are reported for both the AFB_1 *exo*- and *endo*-epoxide–GSH conjugates. Structural assignments were made by comparison with AFB_1 *exo*- and *endo*-epoxide–ethanethiol conjugates, for which nuclear Overhauser effects were measured to establish relative configurations. The *endo*-epoxide was found to be a good substrate for GSH conjugate formation in rat liver cytosol while mouse liver cytosol conjugated the *exo*-epoxide almost exclusively. Human liver cytosol conjugated both epoxide isomers to much lower extents than did cytosols prepared from rats or mice. Purified rat GSH *S*-transferases catalyzed the formation of the AFB_1 *exo*-epoxide–GSH conjugate in the order 1-1 ~ 4-4 ~ 3-3 > 2-2 > 4-6 (7-7 and 8-8 did not form the *exo*-epoxide–GSH conjugate at levels above the nonenzymatic rate). The only rat GSH *S*-transferases that conjugated the *endo*-epoxide were 4-4 and 4-6, with 4-4 being the more active. Purified human GSH *S*-transferases catalyzed the conjugation of the *exo*-epoxide in the order M1a-1a > A1-1 > A2-2 (P1-1 and M3-3 did not have detectable activity), while only GSH *S*-transferase M1a-1a conjugated the *endo*-epoxide. These results are important in the extrapolation of work with animal models to human risk assessment. Further studies on the biological activity of AFB_1 *endo*-epoxide will be required to evaluate its significance; however, the GSH and mercapturic acid derivatives of the *endo*- as well as the *exo*-epoxide should be considered in animal and human studies.

Aflatoxins are of concern in many parts of the world because of the widespread contamination of foodstuffs through mold infestation. AFB_1[1] is one of the most commonly found aflatoxins; it is a potent liver carcinogen in experimental animals and probably in man (*6–10*). AFB_1 shows a considerable species difference in its tumorigenicity, with mice being much more resistant than rats. The tumorigenicity is thought to be well correlated with levels of AFB_1-derived DNA adducts, in the absence of confounding variables such as hepatitis B virus infection (*10–12*).

At least part of the species variation in susceptibility is due to differences in metabolism of AFB_1 (*12, 13*). Oxidation of AFB_1 is known to be necessary to produce its genotoxic properties (*13–15*). The critical initial lesion appears to be 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 (*16, 17*). This adduct is formed in the reaction of DNA with AFB_1 *exo*-epoxide (*18*), derived from the action of cytochrome P-450 (*19*). In humans the rate of formation of AFB_1 *exo*-epoxide varies considerably among individuals, due in large part to the role of cytochrome P-450 3A4 in the oxidation reaction and variation in its level of expression (*20*). As in the case of the cytochrome P-450 enzymes, the GSH *S*-transferases can be important in both detoxication and bioactivation reactions (*21–23*). GSH *S*-transferase is now recognized to be a multigene family containing many enzymes with individual patterns of gene regulation and catalytic specificity (*4, 5, 23, 24*). The resistance of mice to AFB_1 is thought to be related to the high GSH *S*-transferase activity with AFB_1 *exo*-epoxide (*25, 26*). Increased AFB_1 *exo*-epoxide–GSH conjugate formation has also been postulated to account for the ability of compounds such as phenobarbital (*27, 28*), butylated hydroxytoluene (*29*), butylated hydroxyanisole (*30–32*), ethoxyquine (*33*), 1,2-dithiole-3-thione (*34*), and Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-one] (*34*) to provide protection against AFB_1 toxicity and carcinogenesis.

AFB_1 *exo*-epoxide–GSH conjugate formed by mouse liver cytosol was characterized by Moss et al. (*35*) and reported to have trans configuration about the 8,9 bond

* Address correspondence to this author.
† Department of Chemistry and Center in Molecular Toxicology, Vanderbilt University.
‡ Cancer Research Campaign Molecular Toxicology Group, University College and Middlesex School of Medicine.
§ Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University.

[1] Abbreviations: AFB_1, aflatoxin B_1; GSH, glutathione; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; FPLC, fast protein liquid chromatography; COSY, correlated (NMR) spectroscopy; NOE, nuclear Overhauser effect. For discussion of individual forms of GSH *S*-transferase, see refs 1–5.

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of the aflatoxin moiety. A so-called 4-4 (α-class)[2] GSH *S*-transferase has been suggested to be the most active in conjugating AFB_1 *exo*-epoxide in mice (*36*, *37*). However, in rats and humans it is not presently clear which GSH *S*-transferases are most active. Among a series of rat GSH *S*-transferases tested, a 1-1 (α-class) enzyme was found to be the most active (*23*, *38*, *39*). Others have also considered this problem (*40*, *41*); to date all of these studies have been limited in that the synthetic substrate AFB_1 *exo*-epoxide was not used directly but instead was generated by microsomal oxidation of AFB_1, thus making the substrate concentration difficult to control accurately.

We have recently described the chemical synthesis of AFB_1 *endo*-epoxide and its enzymatic formation by human and rat microsomes (*42*). This stereoisomer of the *exo*-epoxide is formed in lesser amounts, and its reactivity with DNA has not been investigated. However, it may contribute to the overall toxicity of AFB_1 and therefore deserves consideration. The availability of synthetic *exo*- and *endo*-epoxides (*18*, *42*) has allowed direct experiments to be carried out on the catalytic efficiency of rat and human liver cytosolic GSH *S*-transferases. These studies have revealed the formation of a novel stereoisomer of the known AFB_1 *exo*-epoxide-GSH conjugate arising from the *endo*-epoxide isomer. The structure of the novel AFB_1 *endo*-epoxide-GSH conjugate is detailed in this resport as well as the enzymatic formation of it and AFB_1 *exo*-epoxide-GSH by rat and human GSH *S*-transferases. The activity of the latter enzymes was a point of particular concern since some reports have suggested that human cytosol is devoid of GSH *S*-transferase activity toward AFB_1 *exo*-epoxide (*25*, *41*, *43*)—a virtual lack of such activity in human liver would have serious implications in risk assessment and also influence the validity of intervention strategies such as GSH *S*-transferase induction.

Experimental Procedures

Chemicals. AFB_1 (Sigma Chemical Co., St. Louis, MO) was quantitated by UV spectroscopy (ϵ_{362} = 21.8 mM^{-1} cm^{-1}) and then treated with dimethyldioxirane to form a 10:1 mixture of AFB_1 *exo*-epoxide/AFB_1 *endo*-epoxide (*18*, *42*). The mixture of epoxides was stored in anhydrous $(CH_3)_2CO$ at -20 °C. The epoxides are stable for many months if the $(CH_3)_2CO$ is rigorously anhydrous. The epoxides were checked by NMR for hydrolysis products just prior to use. Pure *exo*-epoxide could be prepared by crystallization from dry $(CH_3)_2CO/CH_2Cl_2$ (1:1 v/v). The *endo*-epoxide became enriched in the mother liquor from the crystallization. NMR experiments were carried out on a Bruker AM 400 or AC 300 spectrometer (Bruker, Billerica, MA). UV measurements were obtained on a Cary 2300 spectrophotometer (Varian, Walnut Creek, CA). FAB mass spectra were obtained on a VG 70-250 instrument (VG Instruments, Manchester, U.K.).

Enzymatic Synthesis of AFB_1 *exo*-Epoxide-GSH Conjugate. Mouse liver cytosol (10 mg mL^{-1}) was stirred in 50 mM sodium phosphate buffer (pH 7.4) containing 5 mM GSH; AFB_1 epoxide [250 μg of a 10:1 *exo*:*endo* mixture in 0.5 mL of $(CH_3)_2CO$] was added in 5 aliquots over 5 min to 10 mL of the cytosolic system. HCO_2H (2 mL of a 0.4 M aqueous solution) was added to precipitate cytosolic protein. The mixture was centrifuged and the supernatant was filtered (0.4 μm) before loading the sample onto an Alltech octadecylsilyl HPLC column (C18, 10 × 250 mm, 10 μm, Alltech, Deerfield, IL) via the pump head of the chromatograph. After the entire sample was loaded, a linear gradient of 0-40% CH_3CN in H_2O (v/v) over a period of 40 min was applied (3 mL min^{-1}). The AFB_1 *exo*-epoxide-GSH conjugate eluted 18 min after beginning the gradient (eluted at ~18% CH_3CN, v/v). The collected fractions were pooled and dried in vacuo to remove CH_3CN, and the remaining H_2O was removed by lyophilization. The resulting yellow solid (75 μg, 0.12 mmol, 39% yield) was dissolved in ^2H_2O—the ^1H NMR spectrum (Figure 1) closely matched the reported spectrum (*35*): δ 2.08 (m, 2 H, Glu β), 2.47 (m, 2 H, Glu γ), 2.59 (m, 2 H, H-2a,2b), 2.82 (dd, 1 H, Cys β_a, J = 8.6 and 14.2 Hz), 3.16 (dd, 1 H, Cys β_b, J = 5.8 and 14.3 Hz), 3.30 (m, 2 H, H-3a,3b), 3.66 (t, 1 H, Glu α, J = 6.2 Hz), 3.74 (d, 2 H, Gly α, J = 2.9 Hz), 3.99 (s, 3 H, 4-OCH_3), 4.11 (d, 1 H, H-9a, J = 5.8 Hz), 4.57 (dd, 1 H, Cys α, J = 5.7 and 8.6 Hz), 4.62 (s, 1 H, H-9), 5.52 (s, 1 H, H-8), 6.56 (s, 1 H, H-5), 6.76 (d, 1 H, H-6a, J = 5.8 Hz). UV λ_{max} = 364, 266 nm (the value ϵ_{362} = 21.8 mM^{-1} cm^{-1} for AFB_1 was routinely used to quantify solutions).

Enzymatic Synthesis of AFB_1 *endo*-Epoxide-GSH Conjugate. AFB_1 epoxide [3 mg, 10:1 *exo*:*endo*, in 0.75 mL of $(CH_3)_2CO/(CH_3)_2SO$, 4:1 v/v] was added dropwise to 15 mL of rat liver cytosol (21 mg of protein mL^{-1}, 5 mM GSH) over a 5-min period with stirring at room temperature. CH_3CO_2H (0.5 mL of a 2 M aqueous solution) was added, and the solution was centrifuged to remove precipitated protein. The supernatant was dried using a Speed Vac concentrator (Savant, Farmingdale, NY); the residue was redissolved in 5 mL of H_2O and filtered (0.45 μm). An HPLC separation was then carried out using an Alltech semipreparative octadecylsilyl column as described above for the enzymatic synthesis of the AFB_1 *exo*-epoxide-GSH conjugate. A mixture of the *exo*-epoxide-GSH and *endo*-epoxide-GSH conjugate isomers was collected as a single fraction and was dried using a Speed Vac concentrator. The residue was dissolved in 1 mL of H_2O and purified by analytical HPLC incorporating an Alltech Econosphere octadecylsilyl cartridge (4.6 × 250 mm) and the following solvents: (A) 20 mM $NH_4CH_3CO_2$ (pH 4.0) and (B) CH_3CN/CH_3OH, 1:1 v/v. Isocratic elution at 1.5 mL min^{-1} with a mixture of 87% A and 13% B (v/v) gave almost base-line separation of the stereoisomeric conjugates. Typical retention times under these conditions were 22.5 min for the AFB_1 *exo*-epoxide-GSH conjugate and 25.0 min for the AFB_1 *endo*-epoxide-GSH conjugate. The collected fractions were dried again using the Speed Vac concentrator, and the residue was subjected to HPLC separation a second time to ensure purity of the *endo*-epoxide-GSH conjugate. The fraction collected from the second HPLC separation was dried and then lyophilized twice from ^2H_2O before ^1H NMR spectroscopy. This procedure yielded ~200 μg of the AFB_1 *endo*-epoxide-GSH conjugate. ^1H NMR (^2H_2O; Figure 1): δ 2.16 (m, 2 H, Glu β), 2.54 (m, 2 H, Glu γ), 2.66 (m, 2 H, H-2a,2b), 3.10 (dd, 1 H, Cys β_a, J = 8.4 and 14.5 Hz), 3.18 (dd, 1 H, Cys β_b, J = 4.9 and 14.5 Hz), 3.49 (m, 2 H, H-3a,3b), 3.74 (s, 1 H, Glu α), 3.77 (t, 2 H, Glu α, J = 6.4 Hz), 4.00 (s, 3 H, 4-OCH_3), 4.42 (dd, 1 H, H-9a, J = 5.9 and 8.3 Hz), 4.49 (t, 1 H, H-9, J = 8.3 Hz), 4.66 (dd, 1 H, Cys α, J = 4.9 and 8.3 Hz), 4.86 (d, 1 H, H-8, J = 8.3 Hz), 6.55 (d, 1 H, H-5, J = 5.9 Hz), 6.67 (s, 1 H, H-6a). UV (10 mM sodium phosphate, pH 6.5) λ_{max} = 363, 266 nm; (10 mM sodium phosphate, pH 10) λ_{max} = 363, 265 nm.

Chemical Synthesis of AFB_1 *exo*-Epoxide-GSH and AFB_1 *endo*-Epoxide-GSH Conjugates. GSH (200 mg, 0.065 mmol) was stirred in 2 mL of CH_3OH, and 50 mg of Na metal (2.2 mmol) was added. The GSH quickly dissolved, and then 1.5 mg of AFB_1 epoxide (2:1 exo:endo in 0.75 mL of CH_2Cl_2) was added to the solution. The solution immediately became yellow and after 1 min was neutralized with ~1.5 mL of 1 M aqueous CH_3CO_2H. The solvent was removed in vacuo with a Speed Vac concentrator. The residue was dissolved in 4 mL of H_2O and clarified with the use of a 0.4-μm filter. Purification by HPLC (vide supra) gave 70% and 50% yields, respectively, of the *exo*- and *endo*-epoxide conjugates as determined by HPLC peak areas in the chromatogram. The collected fractions were dried using the Speed Vac

[2] Recently, a systematic nomenclature has been adapted for the human GSH *S*-transferases (*4*) but not for other species. Unfortunately, some of the trivial names are confusing—the mouse "4-4" enzyme mentioned here is in the α class and is not to be confused with the rat enzyme termed 4-4 and in the μ gene family (*5*). These nomenclature conventions (*4*, *5*) are used in this paper.

concentrator, and the residues were lysophilized twice from ^2H_2O before obtaining ^1H NMR spectra in ^2H_2O. ^1H NMR spectra for the AFB_1 epoxide–GSH conjugates which had been prepared chemically were identical to spectra obtained from the enzymatically produced AFB_1 epoxide–GSH conjugates.

Synthesis of the Ethanethiol Conjugates of AFB_1 *exo*- and *endo*-Epoxides. Na metal (50 mg) was dissolved in a solution of ethanethiol (100 μL) in 0.5 mL of CH_3OH. AFB_1 epoxide (5 mg, 10:1 exo:endo, in 0.4 mL of CH_2Cl_2) was added to the solution, and the mixture was stirred for 10 min at room temperature. Sodium phosphate buffer (5 mL, 100 mM, pH 4.3) was then added to the reaction mixture. The resulting solution was extracted twice with 5 mL of CH_2Cl_2. The organic layer was retained, and the CH_2Cl_2 was removed in vacuo. The residue was then dissolved in 0.5 mL of CH_3OH, and reverse-phase HPLC was carried out to purify the mixture of products using an Alltech semipreparative octadecylsilyl column (10 × 250 mm), with the elution solvent being $H_2O/CH_3CN/CH_3OH$, 2:1:1 v/v. A flow rate of 3 mL min^{-1} gave retention times of 11.2 and 13.1 min for AFB_1 *exo*-epoxide–ethanethiol and AFB_1 *endo*-epoxide–ethanethiol conjugates, respectively. A second round of reverse-phase HPLC using the same conditions was required for purification of the ethanethiol conjugate of the *endo*-epoxide. The collected products were dried using a Speed Vac concentrator, and ^1H NMR spectra were obtained. FAB mass spectrometry gave an apparent molecular ion (MH^+) at *m/z* 391 for each AFB_1–ethanethiol conjugate. ^1H NMR (C^2HCl_3): (exo) δ 1.23 (t, 3 H, SCH_2CH_3, J = 7.3 Hz), 2.58 (m, 1 H, SC*H*), 2.62 (m, 2 H, H-2,2′), 2.70 (m, 1 H, SC*H*′), 3.40 (m, 2 H, H-3,3′), 3.94 (s, 3 H, 4-OCH_3), 4.07 (d, 1 H, H-9a, J = 6.0 Hz), 4.62 (s, 1 H, H-9), 5.43 (s, 1 H, H-8), 6.33 (s, 1 H, H-5), 6.65 (d, 1 H, H-6a, J = 6.0 Hz); (endo) δ 1.30 (t, 3 H, SCH_2CH_3, J = 7.3 Hz), 2.58 (m, 1 H, SC*H*), 2.62 (m, 2 H, H-2,2′), 2.70 (m, 1 H, SC*H*′), 3.40 (m, 2 H, H-3,3′), 3.94 (s, 3 H, 4-OCH_3), 4.28 (dd, 1 H, H-9a, J = 6.2 and 8.2 Hz), 4.46 (s, 1 H, H-9, J = 8.3 Hz), 4.82 (d, 1 H, H-8, J = 8.3 Hz), 6.38 (s, 1 H, H-5), 6.46 (d, 1 H, H-6a, J = 6.1 Hz).

NOE Difference Experiments. Spectra were recorded using the Bruker AM-400 spectrometer operating at 400 MHz with C^2HCl_3 as solvent. Chemical shifts for the AFB_1 epoxide–ethanethiol derivatives are reported in relation to residual $CHCl_3$ at δ 7.24. For measurement of NOE difference spectra, decoupler power was set at 45L with a 2.5-s irradiation time. Exponential line-broadening of 1.0 Hz was used in the Fourier transformation of difference spectra.

Enzyme Sources. Male rats (200 g) of Sprague-Dawley origin were purchased from Harlan Industries (Indianapolis, IN) and used as a source of cytosol for enzyme assays. Female CD-1 mice (15–20 g) were obtained from Charles River Laboratories (Wilmington, MA). Human liver samples were obtained from organ donors (who met accidental deaths) through Tennessee Donor Services (Nashville, TN). Cytosolic fractions were prepared as described elsewhere (*44*) and frozen at −80 °C.

The levels of some of the individual GSH *S*-transferases were estimated in the rat and human liver cytosol samples using a combination of immunochemical and HPLC assays (*45*). The levels of individual subunits 1, 2, 3, 4, and 8 in rat liver cytosol were approximately 4.9, 8.3, 11.3, 6.9, and 0.8 μg (mg of protein)$^{-1}$, respectively (levels of subunits 6 and 7 are lower; for discussion of subunits 1a and 1b see the text). In 8 human liver samples analyzed the levels of the enzymes A1–1, A2,2, and M1a–1a were in the ranges of 47–171, 36–131, and <3–58 μg (g of wet liver)$^{-1}$, respectively [P1–1 < 1 μg (g of liver)$^{-1}$ in all cases].

Preparation of GSH *S*-Transferases. Rats of Sprague-Dawley origin were purchased from Harlan Olac (Bicester, Oxon, U.K.). Rat GSH *S*-transferases 1–1, 2–2, 3–3, and 4–4 were prepared as described by Beale et al. (*46*), 7–7 was prepared according to Meyer et al. (*47*), and 8–8 was prepared according to Meyer et al. (*48*). Transferase 1–1 is composed of subunits 1a and 1b (*45*), and under control conditions with Sprague-Dawley rats there is about twice as much 1a as 1b. In order to vary the contribution of the two subunits 1, the 1–1 enzyme was prepared from control and 1,2-dithiole-3-thione-induced liver, the latter being elevated in levels of 1b but not 1a (*49*). GSH *S*-transferase 1–1 was partially purified as described (*46*) and finally purified by anion-exchange FPLC (Mono Q 5/5, Pharmacia, Bromma, Sweden) in 30 mM sodium 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.1) containing 3 mM 2-mercaptoethanol and 5% (v/v) glycerol, eluting with a gradient of NaCl. (This extra step was needed because 1,2-dithiole-3-thione-induced subunit 10 contaminates the 1–1 after the standard purification.)

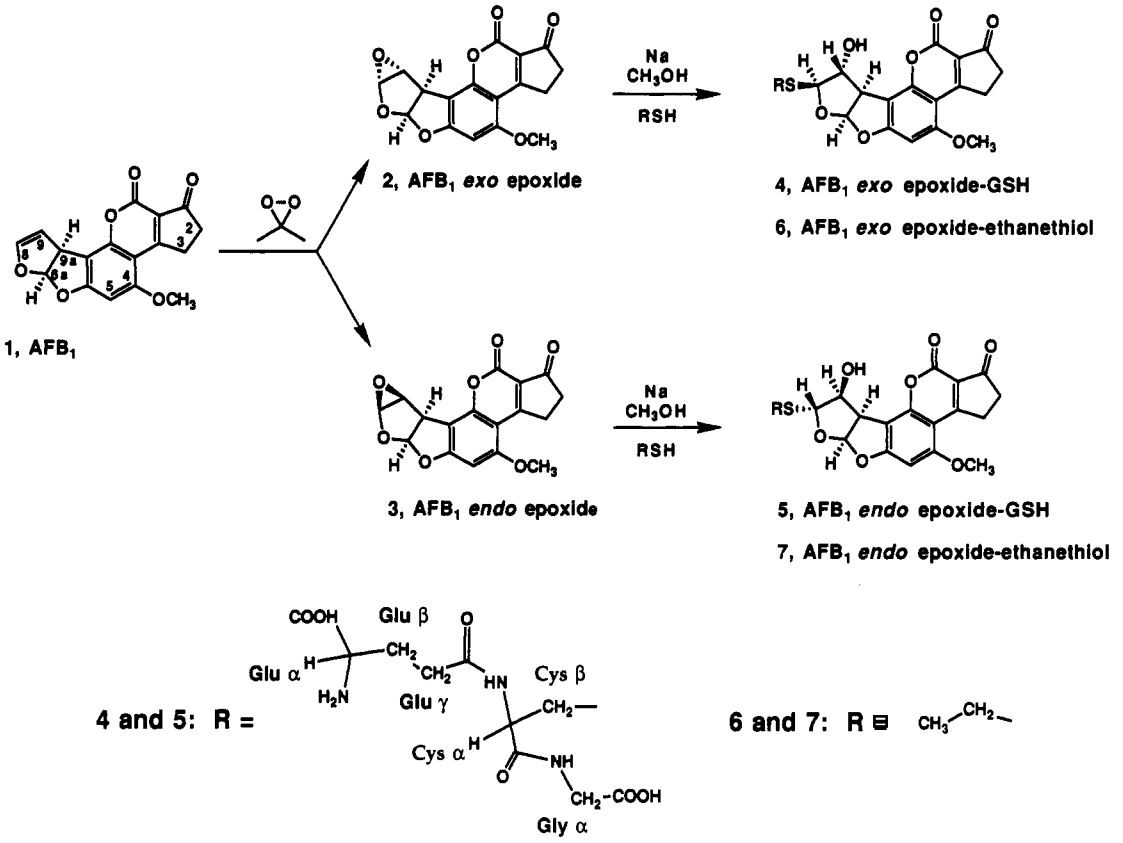
Human α-class GSH *S*-Transferases were obtained by GSH–agarose chromatography of liver cytosol (sample HL 133) and the α-class pool was collected by hydroxylapatite FPLC (HPRT column, Bio-Rad, Richmond, CA) using a gradient of sodium phosphate buffer (pH 6.8). Transferases A1–1 and A2–2 were then separated by anion-exchange FPLC in 30 mM piperazine hydrochloride buffer (pH 9.55) containing 5 mM 2-mercaptoethanol and 10% glycerol (v/v) (*50*). GSH transferases M1a–1a and M3–3 (*51*) were purified from human liver sample HL 133 by sequential GSH–agarose chromatography, hydroxylapatite FPLC, and anion-exchange FPLC (Mono-Q) in 30 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol and 10% glycerol (v/v), eluting with a gradient of NaCl. Human GSH *S*-transferase P1–1 was prepared from kidney by GSH–agarose affinity chromatography followed by anion-exchange FPLC as described for GSH *S*-transferases M1a–1a and M3–3.

GSH *S*-transferases were characterized by reverse-phase HPLC and activity with model electrophilic substrates and, in the case of M1a–1a, isoelectric focusing and primary sequence analysis of CNBr-derived fragments.

GSH *S*-Transferase-Catalyzed Conjugation of GSH with AFB_1 Epoxide. GSH *S*-transferase preparations were dialyzed against 50 mM sodium phosphate buffer (pH 7.4) for 24 h at 4 °C, after which protein content was determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). This dialysis did not alter the activity of the transferases in trial experiments using mouse and rat cytosols. Such enzyme preparations were made 5 mM in GSH immediately prior to assay of conjugation activity.

In a typical experiment, 2 μL of AFB_1 epoxide [10:1 exo:endo, in $(CH_3)_2CO$] was added by microsyringe to 50 μL of enzyme (at 23 °C) while gently mixing with a vortex device to give final concentrations of 50 μM AFB_1 *exo*-epoxide and 5 μM AFB_1 *endo*-epoxide. CH_3CO_2H (20 μL of a 2 M aqueous solution) was added after 15 s to lower the pH, in order to ensure consistent elution of AFB_1 8,9-dihydrodiol and AFB_1–GSH conjugates as well as to precipitate protein. Samples were stored at −20 °C. Thawed samples were centrifuged and then analyzed by reverse-phase HPLC, using an IBM LC/9533 liquid chromatograph incorporating an Alltech Econosphere octadecylsilyl cartridge (5 μm, 4.6 × 250 mm). Solvents used for separation of the two AFB_1 epoxide–GSH conjugates were (A) 20 mM $NH_4CH_3CO_2$, pH 4.0, and (B) CH_3CN/CH_3OH, 1:1 v/v. A linear gradient of a mixture of 90% A and 10% B (v/v) to a mixture of 70% A and 30% B (v/v) over 40 min was used at a flow rate of 1.5 mL min^{-1}. Retention times under these conditions were typically 21 min for AFB_1 *exo*-epoxide–GSH and 22 min for AFB_1 *endo*-epoxide–GSH. Peak identities were confirmed by coinjection with synthetic standards. The hydrolysis product of AFB_1 *exo*-epoxide (AFB_1 8,9-dihydrodiol) eluted at 30 min; other peaks in the HPLC chromatograms in Figures 3–5 were not identified. It should be noted that neither a Whatman octadecylsilyl Partisil cartridge (4.6 × 120 mm, Whatman, Piscataway, NJ) nor a Beckman Ultraphere octadecylsilyl column (4.6 × 250 mm, Beckman, San Ramon, CA) separated the two conjugates under the conditions described here. Detection of the effluent was done using a single-wavelength UV detector at 360 nm, and peak areas were computed using a Hitachi D-2500 Chromato-Integrator (EM Science, Cherry Hill, NJ). The response factor for both AFB_1 epoxide–GSH conjugates was assumed to be equal to that of AFB_1 8,9-dihydrodiol (*26*), which was used as an external standard. AFB_1 8,9-dihydrodiol standards were prepared by adding known amounts of AFB_1 epoxide to aqueous buffer (pH 3.2) (*52*).

Scheme I. Structures and Reactions of AFB_1 and Products



A similar procedure was used with cytosolic fractions. Samples of rat and mouse liver cytosols were made up to 0.5, 1.5, 3.0, and 5.0 mg of protein mL^{-1} in 100 mM sodium phosphate buffer (pH 7.4) containing 5 mM GSH. Human liver cytosol produced detectable levels of AFB_1 epoxide–GSH conjugates only when very high protein concentrations were used (15 mg mL^{-1}). AFB_1 epoxide in $(CH_3)_2CO$ (10 μL of a 10:1 exo:endo mixture at the appropriate stock concentration) was added to 250 μL of cytosol while gently mixing using a vortex device. CH_3CO_2H (50 μL of a 2M aqueous solution) was added to precipitate cytosolic protein, and the samples were stored at −20 °C. Thawed samples were centrifuged prior to HPLC analysis (vide supra).

Results and Discussion

Identification of AFB_1 *endo*-Epoxide–GSH Conjugate. Initial experiments in the development of the assay for measuring relative GSH *S*-transferase activity toward AFB_1 *exo*-epoxide were carried out using a Whatman Partisil octadecylsilyl cartridge for HPLC analysis of AFB_1 *exo*-epoxide–GSH (*51*).[3] This column gave only a single conjugate peak which coeluted with that produced when AFB_1 epoxide was added to mouse liver cytosol. Subsequent experiments carried out using an Alltech Econosphere octadecylsilyl cartridge for the HPLC analysis yielded two peaks when rat liver cytosol was used as the source of GSH *S*-transferase. The earlier eluting peak cochromatographed with chemically and enzymatically (mouse liver cytosol) synthesized AFB_1 *exo*-epoxide–GSH conjugate. A 10:1 mixture of AFB_1 *exo*-epoxide/AFB_1 *endo*-epoxide had been used in these experiments; the possibility was investigated that the novel AFB_1 epoxide–GSH conjugate arose from *endo*-epoxide. When AFB_1 *exo*-epoxide purified by recrystallization was added to rat liver cytosol, the novel AFB_1 epoxide–GSH conjugate was absent. The supernatant from the recrystallization contained a 2:1 mixture of AFB_1 *exo*-epoxide/AFB_1 *endo*-epoxide (*42*). When this mixture was added to rat liver cytosol, formation of the novel conjugate was enhanced, indicating that the novel conjugate arose not from the *exo*-epoxide of AFB_1 but from the *endo*-epoxide.

Characterization of AFB_1 *endo*-Epoxide–GSH Conjugate. The synthesis of AFB_1 *exo*- and *endo*-epoxides and their GSH and ethanethiol conjugates is shown in Scheme I. AFB_1 *exo*-epoxide–GSH (**4**) was synthesized enzymatically from AFB_1 *exo*-epoxide (**2**) and GSH using mouse liver cytosol as the source of GSH *S*-transferase. A chemical synthesis of this conjugate was developed using the thiolate anion of GSH, and the procedure gave good yields. AFB_1 *endo*-epoxide–GSH (**5**) was synthesized enzymatically from AFB_1 *endo*-epoxide (**3**) (from a 10:1 mixture of exo:endo epoxides) and GSH using rat liver cytosol as the source of GSH *S*-transferase. Chemical synthesis from the GSH thiolate anion also gave good yields, and thus chemical synthesis of the conjugates was found to be the most practical route for obtaining pure specimens of these compounds.

The UV spectrum of *endo*-epoxide–GSH conjugate (**5**) did not undergo a bathochromic shift under basic conditions, indicating that conjugation of GSH took place at the 8-carbon of AFB_1 (*54*). The downfield regions of the ^1H NMR spectra of the *exo*- and *endo*-epoxide–GSH conjugates are shown in Figure 1; proton assignments of the *endo*-epoxide–GSH conjugate and the previously identified *exo*-epoxide–GSH conjugate are presented under the Experimental Procedures section. Assignments for *endo*-epoxide–GSH (**5**) were made with the assistance of a COSY spectrum (not shown). The presence of coupling

[3] In light of the present information the values cited in this meeting report are questionable and should be considered superceded by the present study.

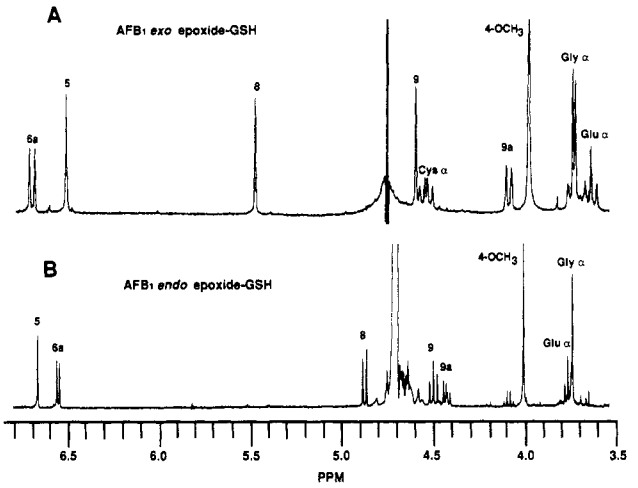


Figure 1. NMR spectra of AFB_1 epoxide–GSH conjugates. (A) ^1H NMR spectrum of AFB_1 *exo*-epoxide–GSH (obtained on Bruker 300 AC spectrometer). (B) ^1H NMR spectrum of AFB_1 *endo*-epoxide–GSH (obtained on Bruker 400 AM spectrometer).

between aflatoxin protons 8 and 9 as well as 9 and 9a in the novel isomer and the lack of detectable coupling between these protons in the previously identified isomer are the principal differences in the spectra of the two conjugates (Figure 1). The coupling pattern for AFB_1 *exo*-epoxide–GSH (**4**) previously identified by Moss et al. (*35*) corresponds to that of the AFB_1–N^7-guanine adduct (*17*), confirming their assignment of a trans configuration. This conclusion is further supported by the mechanism of chemical synthesis of **4** employed in the present study, in which the GSH thiolate anion attacks *exo*-epoxide (**2**) via an S_N2 mechanism. The chemical synthesis of the *endo*-epoxide–GSH conjugate (**5**) can also be expected to occur via an S_N2 mechanism, thus leading to the assignment of trans configuration for the 8- and 9-protons in **5**. The lack of coupling between protons 8 and 9 in *exo*-epoxide–GSH (**4**) as compared with the large coupling constant (8.3 Hz) for these protons in *endo*-epoxide–GSH (**5**) is believed to reflect a difference in the conformations of **4** and **5**.

NOE difference experiments were carried out to substantiate the structural assignments of **4** and **5** and to provide evidence for different conformations of *exo*-epoxide–GSH (**4**) and *endo*-epoxide–GSH (**5**). However, NOE difference experiments performed on **4** and **5** were unsuccessful owing to the presence of the large solvent peak (H_2O). Thus, analogs were sought that would be soluble in organic solvents and would contain a sulfur linkage at the aflatoxin C-8 position. AFB_1 epoxide–ethanediol derivatives were synthesized for this purpose in a manner similar to the chemical synthesis of the AFB_1 epoxide–GSH conjugates. ^1H NMR assignments for AFB_1 *exo*-epoxide–ethanethiol (**6**) and AFB_1 *endo*-epoxide–ethanethiol (**7**) are presented under Experimental Procedures. The chemical shift and coupling patterns of the AFB_1 protons of the two ethanethiol derivatives compare favorably with those of the corresponding AFB_1 epoxide–GSH conjugates. The structures of ethanethiol conjugates **6** and **7** are shown in Scheme I.

NOE difference experiments were carried out on the ethanethiol conjugates; the results are listed in Table I. Each compound shows a strong NOE between protons 6a and 9a due to the cis configuration for these protons. Protons 9 and 9a are trans in **6** and show a small NOE; these two protons are cis in **7**, leading to a larger NOE. Protons 8 and 9 are trans in **6** and **7**. The NOE seen

Table I. NOE Difference Results for AFB_1 Epoxide–Ethanethiol Conjugates 6 and 7

	% NOE							
irradiated	H-6a		H-8		H-9		H-9a	
proton	6	7	6	7	6	7	6	7
H-6a			~0	~0	~0	~0	8.8	8.1
H-8	1.0	~0			4.9	~0	~0	~0
H-9	~0	~0	5.3	~0			3.0	4.9
H-9a	10.5	7.7	~0	~0	2.8	5.3		

between H-8 and H-9 in *exo*-epoxide–ethanethiol (**6**) is of intermediate strength, and this result, in addition to the lack of detectable coupling between protons 8 and 9 in **6**, indicates a torsional angle of ~90° for these protons. A corresponding NOE in *endo*-epoxide–ethanethiol (**7**) is not observed, which suggests these protons are pseudodiaxial, which is consistent with the large vicinal coupling constant (8.3 Hz) (*55*).

Assay Development. Development of an assay for conjugation rates posed special problems. AFB_1 *exo*-epoxide is very unstable in H_2O; the $t_{1/2}$ for hydrolysis has been estimated to be <5 s (*18*, *52*). Therefore, hydrolysis and GSH conjugation are competing reactions. It has not yet been possible to measure the hydrolysis rate precisely and then to express the conjugation rate in units of time. The hydrolysis of AFB_1 *endo*-epoxide has not been investigated; however, the compound is also expected to be hydrolytically unstable. Consequently, relative catalytic efficiencies of cytosols and purified GSH *S*-transferases are expressed as nanomoles of conjugate formed per milligram of protein at a given epoxide concentration. A 10:1 mixture of *exo*- and *endo*-epoxide (*42*) was used for these experiments (procedures for complete purification of AFB_1 *endo*-epoxide have not yet been developed). A small amount of AFB_1 *exo*-epoxide–GSH was formed nonenzymatically; enzymatic yields reported were corrected for this. Nonenzymatic formation of AFB_1 *endo*-epoxide–GSH was not detected due to the very low concentration of *endo*-epoxide used in these experiments.

Comparison of Conjugation Activities in Mouse, Rat, and Human Liver Cytosols. Mouse, rat, and human liver cytosols were analyzed for their abilities to conjugate GSH with the epoxides of AFB_1. Some strains of mice are resistant to the carcinogenic effects of AFB_1 while rats are susceptible, and this difference is thought to be due to efficient scavenging of the *exo*-epoxide by GSH *S*-transferases in mice (*25*, *56*). This view is consistent with the results of experiments described here using synthetic AFB_1 *exo*-epoxide as the substrate. The conjugation efficiency of mouse cytosol was 25-fold greater than that of rat liver cytosol with 30 μM AFB_1 *exo*-epoxide (Figures 2 and 3). Mouse liver cytosol appears to conjugate the *exo*-epoxide isomer almost exclusively while rat liver cytosol conjugates both epoxides, with the *endo*-epoxide being the better substrate (Figures 2 and 3). It should be emphasized that rat liver cytosol formed more *endo*-epoxide–GSH than *exo*-epoxide–GSH even though the *endo*-epoxide was 10-fold lower in concentration than the *exo*-epoxide. Several human liver cytosols were assayed for epoxide conjugation activity. At high cytosolic protein concentration (15 mg mL^{-1}), very small amounts of both epoxide–GSH conjugates were detected which approached the detection limit for the analysis described here. Human liver cytosol does appear to conjugate both AFB_1 epoxide isomers, however, with lower efficiency than rat (Figure 4). Human liver cytosol sample HL136 showed the highest conjugating

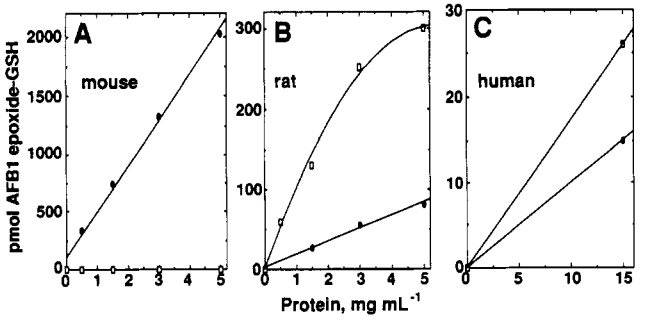


Figure 2. Conjugation of AFB_1 *exo*- and *endo*-epoxides with GSH catalyzed by liver cytosolic fractions. All assays were done with a mixture of AFB_1 *exo*-epoxide (30 μM) and AFB_1 *endo*-epoxide (3 μM). The conjugates derived from both the *exo*- (●) and *endo*- (□) epoxides are shown. (A) Mouse liver cytosol; (B) rat liver cytosol; (C) human liver cytosol (sample HL136).

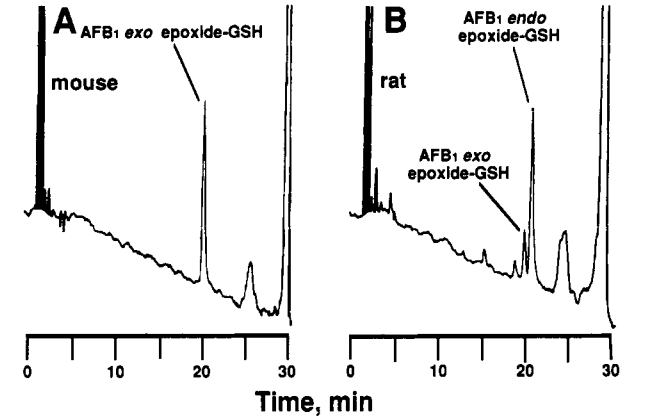


Figure 3. HPLC of products of conjugation of AFB_1 epoxides by mouse and rat liver cytosolic fractions. Shown are the products of a reaction of a mixture of 30 μM AFB_1 *exo*-epoxide and 3 μM AFB_1 *endo*-epoxide with (A) mouse liver cytosol (1.5 mg of protein mL^{-1}) or (B) rat liver cytosol (3.0 mg of protein mL^{-1}). The full scale of the detector was 0.005 absorbance unit.

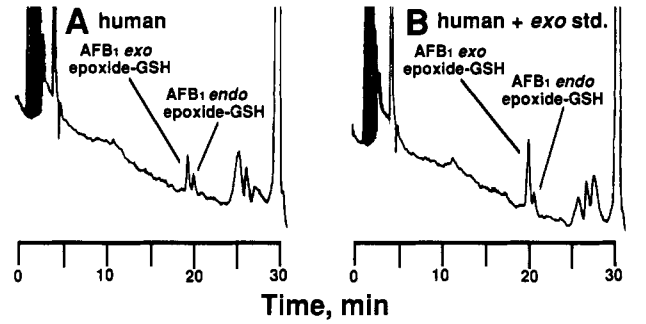


Figure 4. HPLC of products of conjugation of AFB_1 epoxides by human liver cytosolic fraction. Shown are the products of a reaction of a mixture of 30 μM AFB_1 *exo*-epoxide and 3 μM AFB_1 *endo*-epoxide with (A) human liver cytosol (15 mg of protein mL^{-1}) and (B) the same human liver cytosol sample spiked with authentic AFB_1 *exo*-epoxide–GSH conjugate. The full scale of the detector was 0.005 absorbance unit.

activity, and results are shown in Figure 2. With this cytosol sample, conjugation efficiency of AFB_1 *exo*-epoxide was 400 times lower than with mouse liver cytosol and 25 times lower than with rat liver cytosol (on a milligram of protein basis). Conjugation efficiency for AFB_1 *endo*-epoxide was greater with human liver cytosol than with mouse liver cytosol, but ~30 times lower than with rat liver cytosol.

Relative Activity of Rat GSH *S*-Transferases. Results of studies on the formation of GSH conjugates of AFB_1 *endo*- and *exo*-epoxides by purified rat and human GSH *S*-transferases are summarized in Table II. For rats, the order of catalytic activity observed for conjugation of the *exo*-epoxide was 1–1 ~ 4–4 ~ 3–3 > 2–2 > 4–6. No conjugate above the nonenzymatic background was detected with rat transferase 7–7 or 8–8. The order of catalytic activity observed in the conjugation of AFB_1 *endo*-epoxide was 4–4 > 4–6. Conjugation of the *endo*-epoxide isomer was not detected with any of the other rat enzymes. Thus, a μ-class enzyme (4–4) is the most active of those examined, and it conjugates the *endo*-epoxide predominantly (Figure 5). α-Class enzyme 1–1 and μ-class enzyme 3–3 have significant activity and conjugate the *exo*-epoxide isomer exclusively (Figure 5, Table II). A μ-class heterodimer 4–6 was also tested, and this enzyme conjugated both epoxides.

When the specific activities of the individual GSH *S*-transferases were multiplied by the known content in rat liver cytosol, ~1/3 of the AFB_1 epoxide conjugating activity of the cytosol could be accounted for in the known enzymes. The low value may be due to deficiencies in estimation of protein concentrations or loss of catalytic activity during purification. The existence of additional GSH *S*-transferases represents another possibility. In this regard, Hayes et al. (57) have recently reported that GSH *S*-transferase Yc_2–Yc_2, which is closely related to or identical to

Table II. Rank Order of AFB_1 Epoxide–GSH Conjugate Formation Activity by Purified GSH *S*-Transferases

	nmol of conjugate (mg of protein)$^{-1}$	
enzyme	AFB_1 *exo*-epoxide–GSH (4)	AFB_1 *endo*-epoxide–GSH (5)
rat 1–1(1a:1b mixture, 2:1 ratio)[a]	1.7	<0.16
rat 1–1(1a:1b mixture, 1:2 ratio)[b]	1.1	<0.10
rat 2–2	0.4, 0.5[c]	<0.30
rat 3–3	1.3, 1.3	<0.22
rat 4–4	1.2, 1.8	15.8, 14.8
rat 4–6	0.3, 0.2	9.1, 6.2
rat 7–7	<0.43	<0.43
rat 8–8	<0.13	<0.13
human A1–1	0.04, 0.04	<0.01
human A2–2	0.01, 0.02	<0.01
human M1a–1a	0.6, 0.5	1.4, 1.3
human M3–3	<0.14	<0.14
human P1–1	<0.06	<0.06

[a] Prepared from untreated rats. [b] Prepared from 1,2-dithiole-3-thione-treated rats. [c] Duplicate assays were done when two values are reported—the variation was typical.

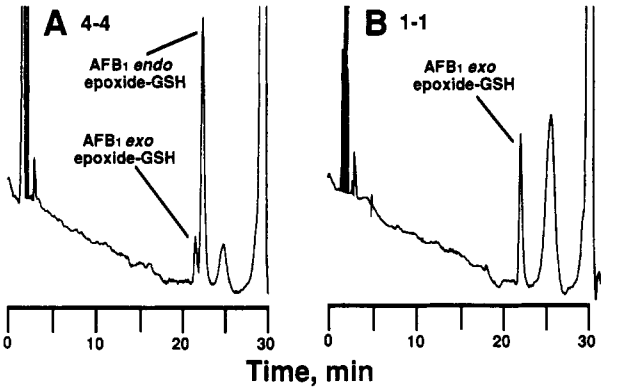


Figure 5. HPLC of products of conjugation of AFB_1 epoxides by rat liver GSH *S*-transferases 4–4 and 1–1. Shown are the products of a reaction of a mixture of 50 μM AFB_1 *exo*-epoxide and 5 μM AFB_1 *endo*-epoxide with (A) transferase 4–4 (0.25 mg of protein mL^{-1}) or (B) transferase 1–1 (0.35 mg of protein mL^{-1}). The full scale of the detector was 0.005 absorbance unit.

subunit 10 (58), is highly active in the conjugation of AFB_1 epoxide. In agreement with this, the elevated activity seen in the DEAE-cellulose-bound portion of a rat liver cytosolic fraction after treatment of rats with 1,2-dithiole-3-thione (vs control rats) is probably at least partially due to the induction of subunit 10.

Selectivity of Human GSH *S*-Transferases. Among the purified human GSH *S*-transferases studied, the observed order of activity for conjugation of the *exo*-epoxide was M1a-1a > A1-1 > A2-2 (Table II). Neither the π-class enzyme P1-1 nor the μ-class enzyme M3-3 produced levels of conjugate above the nonenzymatic levels. The μ enzyme M1a-1a also conjugated the *endo*-epoxide, and the level of reaction was higher than for the *exo*-epoxide. Human GSH *S*-transferases appear to have some ability to conjugate both *exo*- and *endo*-epoxides. In this regard Liu et al. (59) recently reported a correlation between levels of GSH *S*-transferase μ (M1a-1a) in human liver cytosol samples and the inhibition of DNA adduct formation from AFB_1 in NADPH-fortified human liver microsomes. However, the significance of that work is difficult to evaluate in light of the small variation in inhibition observed in the assays (<2-fold).

Conclusions. The described syntheses provide a useful approach to authentic AFB_1 epoxide–GSH conjugates and mercapturic acid standards for biological and epidemiological studies. A convenient assay for the direct measurement of conjugation of GSH with the *exo*- and *endo*-epoxides of AFB_1 has been developed. The studies described here utilized a mixture of the two epoxides as the substrate due to lack of a procedure for purification of the *endo*-epoxide. After procedures for the purification of this epoxide are developed, a more thorough study of GSH *S*-transferase activity using pure epoxides will be warranted. Key findings in this study include a novel GSH conjugate of AFB_1 *endo*-epoxide which was isolated and characterized and identification of GSH *S*-transferases which preferentially conjugate one or the other epoxide. Mouse liver cytosol conjugates the *exo*-epoxide isomer almost exclusively and with greater efficiency than rat and human liver cytosols. Rat liver cytosol conjugates both AFB_1 epoxide isomers, with the *endo*-epoxide isomer serving as the better substrate. Human liver cytosol conjugates both AFB_1 epoxide isomers, however, at very low levels.

The μ-class enzymes (rat 4-4 and 4-6, and human M1a-1a) appear to be capable of conjugating both the exo and endo AFB_1 epoxides, with the endo isomer serving as the better substrate. This observation is consistent with the studies of Armstrong and his associates, who reported that rat GSH *S*-transferase C (4-4) showed a preference to add GSH to the oxirane carbon of the *R* absolute configuration in several arene oxide substrates (60, 61). The α-class enzymes studied appear to conjugate the *exo*-epoxide exclusively. The most active mouse GSH *S*-transferase identified (so-called 4-4 or Ya-Ya; note discordance of the rat enzyme nomenclature) (36, 37)[2] is an α-class transferase, consistent with the trend observed here in that mouse liver cytosol conjugated AFB_1 *exo*-epoxide almost exclusively. The fact that rat transferase 3-3, a μ-class enzyme, conjugated the *exo*-epoxide illustrates that a clear correlation between the class of GSH *S*-transferase and the conjugation of one or the other epoxide isomer may not exist when different animal species are considered. Further experiments are required to determine whether AFB_1 *endo*-epoxide is toxicologically significant. It appears, however, that the *endo*-epoxide is overall a better substrate than the *exo*-epoxide for rat and human liver cytosol GSH *S*-transferases.

It is known that α-class GSH *S*-transferases can be induced in rats by 1,2-dithiole-3-thiones and some other compounds capable of generating electrophiles; this process gives rise to substantial protection against the carcinogenic effects of AFB_1 (28, 32), and it seems clear that this is partly due to induction of subunits having unusually high activity (Yc_2/10). It will be of interest to determine if other rat GSH *S*-transferases with high conjugating activity can also be induced. The extremely low level of GSH *S*-transferase activity for AFB_1 epoxides exhibited by human liver cytosol would suggest that humans may not be suitable for such cancer prevention strategies; however, the effects of induction may even be more marked than in rats—further, inducible human GSH *S*-transferases with high activity for AFB_1 epoxides may yet be found. The in vivo significance of our results may be considered once assays for the excreted AFB_1 epoxide–GSH conjugates and their mercapturic acid derivatives have been developed which can be employed in epidemiological studies.

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