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IN VITRO FORMATION OF GLUTATHIONE CONJUGATES OF THE DIMETHYLESTER OF BILIRUBIN

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Abstract—Rat hepatic microsomes catalyzed the formation of two distinct glutathione conjugates of bilirubin dimethylester (DMB). The two conjugates were identical to those isolated from the bile of Gunn rats infused with DMB. The microsomal reaction was dependent on NADPH, oxygen and glutathione and was inhibited by nitrogen and the cytochrome P450 inhibitors metyrapone, 1-benzylimidazole, and α-naphthoflavone. Conjugate formation was inducible with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) but not phenobarbital pretreatment. The rate of formation of conjugates was not affected by washings of the microsomal pellet or by the presence of superoxide dismutase and/or catalase. Cation fast atom bombardment mass spectrometry (FAB/MS) of the conjugates indicated a molecular ion of 937 atomic mass units (amu). Fragmentation revealed a loss of 307 amu, consistent with glutathione, and a residual mass of 629 amu suggesting a hydroxylated derivative of DMB (612 amu). Cation FAB/MS/MS of conjugates formed *in vitro* under an atmosphere of oxygen-16 and oxygen-18 demonstrated the incorporation of molecular oxygen by a difference of 2 amu in the respective molecular ions. Our results suggest that DMB is oxidized by the cytochrome P450 IA gene family to an epoxide intermediate which is then subsequently conjugated with glutathione.

Degradation of protoheme leads to the formation of the open chain tetrapyrrole bilirubin IX-α, and intramolecular hydrogen bonding is responsible for its insolubility in water and physiologic fluids (10^{-14} M) [1]. Biotransformation of the bilirubin molecule to an exportable form normally involves hepatic conjugation with glucuronic acid at either one or both of its propionic side chains. This esterification is catalyzed by bilirubin UDP-glucuronosyltransferases (EC 2.4.1.17) with UDP glucuronic acid as the glycoside donor [2]. Other neutral ester glycosides of bilirubin, including glucose and xylose, have been identified in some mammalian bile [3–5]. However, under normal conditions more than 90% of bilirubin is conjugated and excreted in the bile as the water-soluble mono- and diglucuronide esters [6].

A complete deficiency of bilirubin UDP-glucuronosyltransferase activity results in severe unconjugated hyperbilirubinemia as observed with the autosomal recessive disorders, Crigler–Najjar syndrome Type 1 of humans and the Gunn rat [7, 8]. The bilirubin UDP-glucuronosyltransferase has been reported to be missing in both the Gunn rat and Type I Crigler–Najjar syndrome patients [9–11]. A mutated inactive enzyme has also been reported in the latter [11].

An alternative excretory pathway for bilirubin has been reported by Kapitulnik and Ostrow [12]. They quantitated a 60% decrease in plasma bilirubin concentration and a reduction in the total bilirubin pool of 89% in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated Gunn rats. Folch solvent partitioning of the bile demonstrated an increased output of polar derivatives of bilirubin which were not glucuronic acid conjugates, but the identity of these polar derivatives has not been adequately determined. Odell *et al.* [13] infused Gunn rats with the dimethylester of bilirubin (DMB), a bilirubin analog, and described the presence of four thioether glutathione conjugates in the collected bile [14]. Diazosulfanilate derivatization of two of these pigments demonstrated the glutathione conjugation to have occurred on the exovinyl azodipyrrole, and 1-D NMR of the conjugated tetrapyrroles showed the exovinyl protons to be missing. We now provide evidence from *in vitro* studies that formation of two of these glutathione conjugates of DMB involves the cytochrome P450 IA family which forms epoxide intermediates prior to the glutathione conjugation.

METHODS

Materials. NADPH, dithiothreitol (DTT), glutathione, bis(*p*-nitrophenyl)phosphate, EDTA, formic acid, Trizma-7.8, metyrapone, superoxide dismutase, catalase, potassium citrate, bilirubin, and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from the Sigma Chemical Co. (St. Louis, MO). 1-Benzylimidazole, 1,4-dioxane, and α-naphthoflavone were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Phenobarbital (PB) was obtained from Elkins–Sinn Inc. (Cherry Hill, NJ) and Coomassie Protein Assay Reagent from

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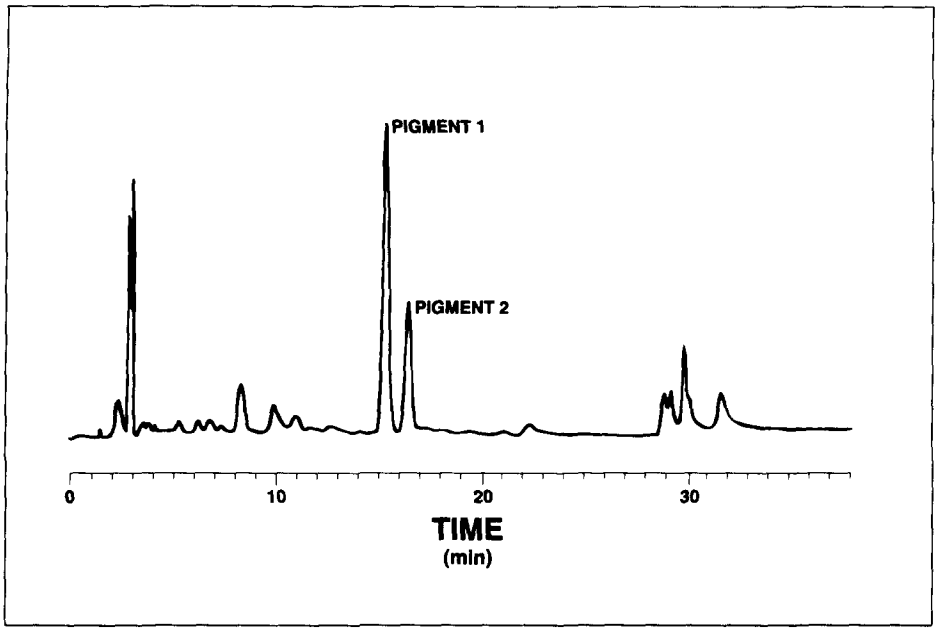


Fig. 1. Reversed-phase HPLC chromatograph of hepatic microsomes from Gunn rats pretreated with TCDD (see Methods). Pigment 1 eluted at 15.3 min and pigment 2 at 16.5 min.

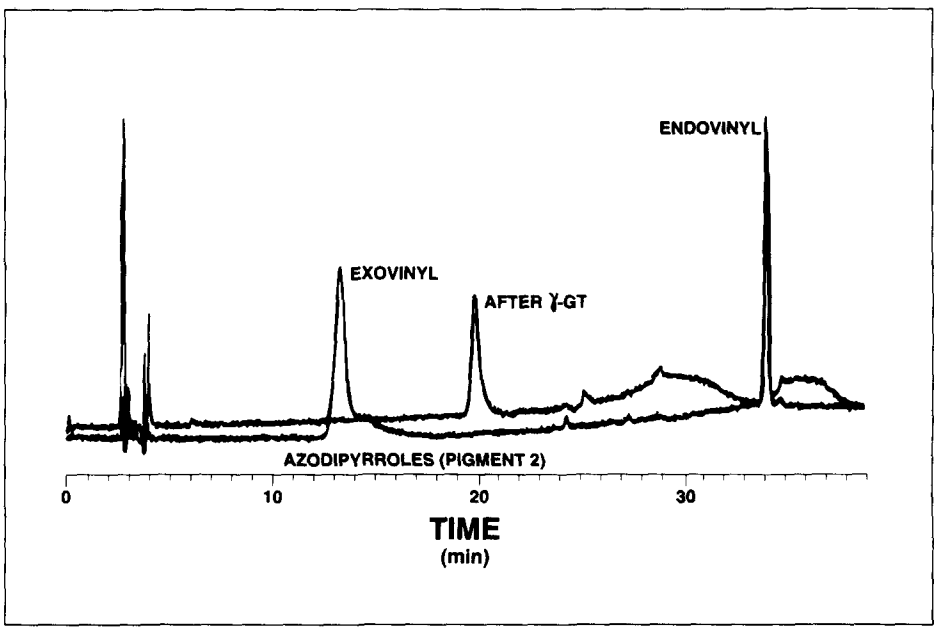


Fig. 2. Reversed-phase HPLC chromatograph of the diazosulfanilate derivative of pigment 2 before and after incubation with γ-glutamyltranspeptidase.

Pierce (Rockford, IL). Other chemicals were purchased from various sources at the highest quality available. DMB was synthesized as reported previously [15]. Oxygen-16 and oxygen-18 was purchased from MSD Isotopes (St. Louis, MO). TCDD was a gift from Dr. Alan Poland, Madison, WI. Pure cytochrome P450c was a gift from Dr. Colin Jefcoate, Madison, WI.

Animals. Female Gunn rats (jj) weighing 200–300 g were used throughout this study. A breeding

Table 1. Effects of various cofactors and inhibitors on the formation of the glutathione conjugates of the dimethylester of bilirubin by rat liver microsomes

Cofactor or inhibitor	Activity* (% of control)
−NADPH	3, 3
− O_2 (4 min N_2 purge)	11 ± 4‡
− Glutathione	16 ± 7†
+Metyrapone (150 μM)	56 ± 8†
+1-Benzylimidazole (150 μM)	7 ± 3‡
+α-Naphthoflavone (150 μM)	3 ± 3‡

* The formation of glutathione conjugate of dimethylester bilirubin was determined by measuring the conversion of bilirubin dimethylester (116 μM) to its conjugated forms. The results are means ± SD for three experiments except for "−NADPH" where the results are shown for two experiments. The control value for microsomal enzyme activity was 390 ± 10 pmol DMB–glutathione/mg microsomal protein/min. Microsomes are from rats pretreated with TCDD.

† Significantly different from control, $P < 0.01$ (Student's *t*-test).

‡ Significantly different from control, $P < 0.001$ (Student's *t*-test).

Table 2. Effects of superoxide dismutase and catalase on the formation of pigments 1 and 2 in Gunn rat liver microsomes from TCDD-pretreated animals

Treatment	Specific activity* (pmol/min/mg protein) Pigment 1	Pigment 2
Control	354 ± 21	148 ± 10
Superoxide dismutase (100 units/mL)	354 ± 33	146 ± 9
Catalase (1100 units/mL)	335 ± 13	142 ± 16
Superoxide dismutase and catalase	367 ± 25	153 ± 8

* The formation of the glutathione conjugates of bilirubin dimethylester was determined by measuring the conversion of bilirubin dimethylester (116 μM) to its conjugated forms. The results are means ± SD (three separate animals).

Table 3. Effects of enzyme inducers on the formation of pigments 1 and 2 in Gunn and Wistar rat liver microsomes

Strain	Pretreatment	Specific activity* (pmol/min/mg protein)
Gunn	Control	22 ± 12
	Phenobarbital	53 ± 32
	TCDD	348 ± 52
Wistar	Control	13 ± 2
	TCDD	289 ± 96

* The formation of the glutathione conjugates of bilirubin dimethylester was determined by measuring the conversion of bilirubin dimethylester (132 μM) to its conjugated forms. The results are means ± SD (three separate animals).

colony of Gunn rats has been maintained at the Animal Care facility of the UW Clinical Sciences Center since 1976. Wistar rats were obtained from the Harlan Sprague–Dawley Co. (Indianapolis, IN). All rats were allowed unlimited access to food (Lab Blox, Wayne Rodent Blox) and water. Lighting was cycled 12 hr daily. Rats from both strains were pretreated with either phenobarbital (0.5 g/L in their drinking water for 11 days) or TCDD (single 10 μg/kg i.p. injection, in 1,4-dioxane) 48–72 hr prior to being killed. Control rats received 1,4-dioxane in a volume equivalent to that in which TCDD was dissolved.

Preparation of microsomes. Rats were starved for 24 hr prior to being killed. Liver homogenates (25%, w/v) were made in 0.25 M sucrose, 25 mM KCl, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.55, and centrifuged at 10,000 *g* for 20 min. The supernatant fractions were centrifuged at 105,000 *g* for 60 min. Washings of the microsomal pellet, where indicated, were done twice by resuspension in 10% potassium citrate, 25 mM KCl, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.55, in a volume equivalent to the supernatant and centrifuged at 105,000 *g* for 45 min.

Incubation conditions. The standard incubation conditions consisted of 1 mg/mL microsomal protein, 0.13 mM DMB [dissolved in dimethyl sulfoxide (DMSO), final concentration of 4% (v/v)], 1 mM NADPH, 2 mM glutathione, 0.5 mM bis(*p*-nitrophenyl)phosphate, 1 mM DTT, and 1 mM EDTA in a total volume of 500 μL of 0.1 M Tris buffer, pH 7.5. The reaction mixture was preincubated at 37° for 2 min prior to the addition of NADPH to start the reaction. Reactions were stopped by the addition of 500 μL of 2 mM ascorbate in ethanol (4°) and centrifuged for 4 min at 8000 rpm to pellet the protein. The supernatants were passed through 0.2 μm filters and 375 μL was used for analysis of conjugate formation by HPLC. All steps were performed in the dark.

The microsomal incubation of DMB described above was carried out on a preparative scale for the mass spectrometric analysis of the pigments formed *in vitro* in the oxygen-16 and oxygen-18 atmospheres. The incubation mixtures were placed in sealed vials (3 mL to each) from which the air was evacuated by vacuum and replaced with nitrogen gas. This was repeated three times before exposure to the particular oxygen isotope (250 mL). After incubation for 30 min, the reaction was stopped by addition of 2 mM ascorbate in ethanol. The incubation mixture was centrifuged and the supernatant filtered and analyzed by HPLC as before. The two glutathione conjugates were collected together on ice in the dark and stored at −70° overnight. Samples were then lyophilized and analyzed by mass spectrometry.

Treatment of DMB–glutathione conjugates with γ-glutamyltranspeptidase. The two glutathione conjugates were collected from the HPLC on ice in the dark. The pigments were frozen overnight at −70°, lyophilized, and redissolved in 1:1 methanol:0.1 M acetic acid. Both pigments were incubated for 30 min with γ-glutamyltranspeptidase (100 units/mL) at 37°. The dipyrrolic azosulfanilates of these pigments were

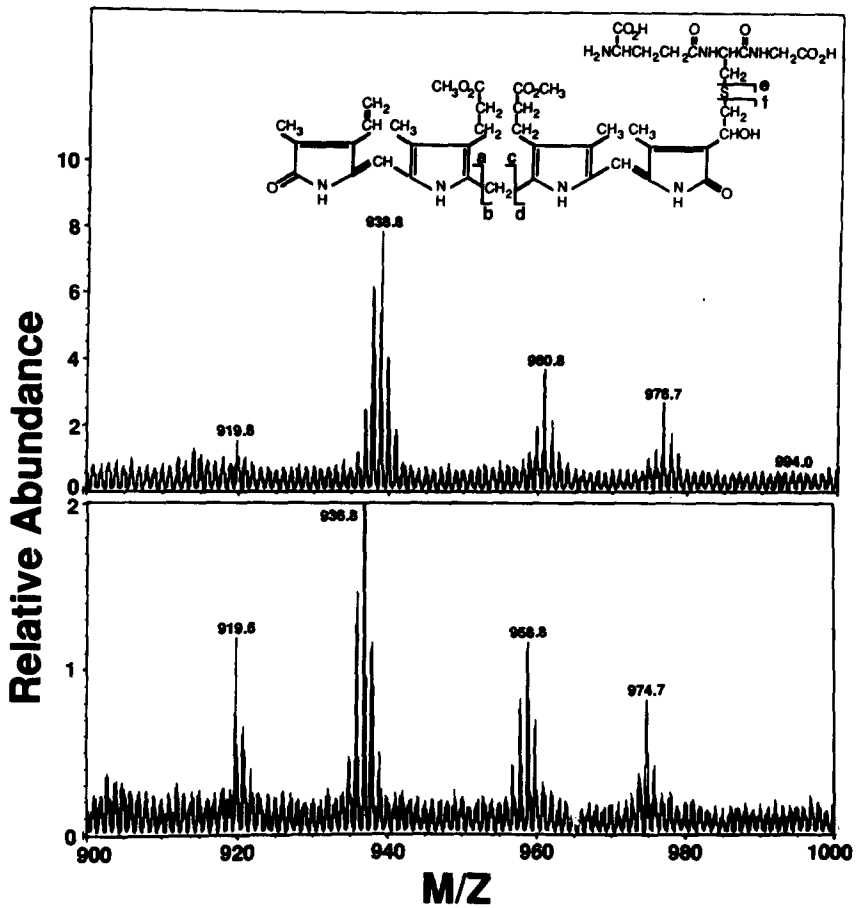


Fig. 3. Cation FAB mass spectra of the glutathione conjugates formed *in vitro* in either the oxygen-18 (top) or oxygen-16 (bottom) atmosphere: MS-1 scan. The two pigments exhibited a difference of 2 amu in the molecular ions $[M + H]^+$ 936.8 and 938.8 as well as the concomitant $[M + Na]^+$ and $[M + K]^+$ ions. This is consistent with the metabolic incorporation of one mole of oxygen in the conjugate from either the oxygen-18 or oxygen-16 atmosphere. The signal at 919 is from the matrix.

Table 4. Effect of washing the microsomal pellet on the formation of pigments 1 and 2

	Specific activity† (pmol/min/mg protein)	
Washings*	Pigment 1	Pigment 2
None	274 ± 10	117 ± 6
One	314 ± 13	131 ± 5
Two	296 ± 12	125 ± 5

* Washing buffer was composed of: 10% potassium citrate, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 mM Tris, pH 7.55.

† The formation of the glutathione conjugates of bilirubin dimethylester was determined by measuring the conversion of bilirubin dimethylester (132 μM) to its conjugated forms. The results are means ± SD (three separate experiments).

prepared before and after the γ-glutamyl-transpeptidase incubation as previously reported [16]. These derivatives were then analyzed by HPLC.

HPLC analysis. The HPLC analysis of the pigments formed *in vitro* was performed as previously described except that ammonium formate was used in place of ammonium acetate [14]. The amount of glutathione conjugates formed was quantitated from the integrated area at 450 nm applying a millimolar extinction of 60 as previously described for the glycoside conjugates of bilirubin [16].

Mass spectrometry. The mass spectrometric analyses were performed as previously described [14] at the laboratory of Dr. Catherine Fenselau of the Department of Chemistry and Biochemistry, University of Maryland.

RESULTS

Formation of DMB–glutathione. The incubation of DMB with rat liver microsomes in the presence of NADPH, glutathione, O_2, and bis(*p*-nitrophenyl)phosphate, to inhibit demethylation of DMB [17, 18], led to the formation of two distinct metabolites, pigments 1 and 2 (Fig. 1). Complete inhibition of demethylation by bis(*p*-nitrophenyl)-phosphate was verified by the absence of free bilirubin in the HPLC chromatograph. These

Table 5. Effect of washing the microsomal pellet on glutathione *S*-transferase activity

	Specific activity* (nmol/min/mg protein)		
	Washings†		
	None	One	Two
Microsomal pellet	352 ± 4	78 ± 8	63 ± 9

* The activity of cytosolic glutathione *S*-transferases was assayed according to the method of Habig *et al.* [19], using 1-chloro-2,4-dinitrobenzene as the substrate. The activity of the cytosolic glutathione *S*-transferases was 2.19 μmol/min/mg protein. The results are means ± SD (three separate experiments).

† Washing buffer was composed of: 10% potassium citrate, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 mM Tris, pH 7.55.

Table 6. Effect of cytosol on the microsomal formation of pigments 1 and 2

	Specific activity* (pmol/min/mg protein)	
Fraction	Pigment 1	Pigment 2
Microsomes + intact cytosol	402 ± 31	166 ± 17
Microsomes + denatured cytosol†	344 ± 30	139 ± 56

* The formation of the glutathione conjugates of bilirubin dimethylester was determined by measuring the conversion of bilirubin dimethylester (132 μM) to its conjugated forms. The results are means ± SD (three separate experiments). The activity of the cytosolic glutathione *S*-transferases was 2.01 ± 0.21 μmol/min/mg protein.

† Cytosol was placed in boiling water bath for 5 min. Proteins were centrifuged out and an equal amount of supernatant was added to the incubate. Final concentration of cytosol was 4 mg/mL.

tetrapyrrolic metabolites formed in the incubation mixtures were identical in their retention times and UV spectra to the exovinyl glutathione conjugates formed *in vivo* [13, 14]. Azosulfanilate derivatization and HPLC analysis of the two pigments were carried out to determine where on the tetrapyrrole conjugation occurs. Comparison to the unconjugated endo- and exovinyl dipyrrolic azosulfanilates of DMB revealed that the conjugation occurs on the exovinyl azodipyrrole [16]. Figure 2 depicts the dipyrrolic azosulfanilate derivatives formed from pigment 2 before and after incubation with γ-glutamyltranspeptidase. Incubation with γ-glutamyltranspeptidase caused a shift of the exovinyl peak to a later time demonstrating that the conjugate was susceptible to this enzyme, consistent with glutathione. The endovinyl azodipyrrole eluted at the same time before and after the incubation and therefore was not susceptible to the enzyme. The formation of conjugates was dependent on, and increased linearly with, protein concentration (data not shown). Subsequent experiments used a protein concentration of 1 mg/mL, at which the formation of conjugates was linear up to 5 min (not shown). The maximum rate of this reaction was observed in the pH range from 7.5 to 8.0 and therefore incubations were performed for 4 min at 37° and pH 7.5.

Inhibition of conjugate formation. The effect of the omission of various reaction components on the formation of DMB–glutathione conjugates is shown in Table 1. The omission of NADPH resulted in complete lack of formation of conjugates. Omission of exogenous glutathione significantly reduced conjugate formation but did not reduce it to zero probably due to residual glutathione present in the microsomal preparations since the pellets were not washed. The addition of typical cytochrome P450 inhibitors such as metyrapone, 1-benzylimidazole, and α-naphthoflavone effectively inhibited the formation of DMB–glutathione conjugates. The reaction was also oxygen-dependent since reactions conducted under a nitrogen atmosphere prevented conjugate formation. The antioxidant enzymes, superoxide dismutase and catalase, apart and together, had little effect on the formation of conjugates (Table 2).

Effects of in vivo *treatment with inducers on DMB–glutathione formation.* The effects of pretreatment of Gunn and Wistar rats with enzyme inducers on DMB–glutathione formation are shown in Table 3. Liver microsomes from both the Gunn and Wistar rats treated with TCDD showed more than a 10-fold increase in the formation of DMB–glutathione conjugates above controls. Microsomes from phenobarbital-pretreated Gunn rats formed the conjugates at a rate that was similar to control microsomes from both species (Table 3).

Mass spectrometric analysis of DMB–glutathione conjugates. Cation fast atom bombardment mass spectrometry (FAB/MS) of the two glutathione conjugates has shown that both pigments have identical molecular ions of 937 atomic mass units (amu). Therefore, subsequent analyses were of both pigments combined. The 937 amu molecular ion is 324 mass units greater than the parent DMB (612 amu). Fragmentation revealed a loss of 307 amu, consistent with glutathione, and a residual mass at 629 amu suggesting a hydroxylated derivative of DMB (629 − 612 = 17 amu). To confirm the presence and incorporation of molecular oxygen in the DMB–glutathione conjugates, hepatic microsomes from TCDD-pretreated rats were incubated under an atmosphere of either oxygen-16 or oxygen-18. Cation FAB/MS/MS of the isolated conjugates from the oxygen-16 and oxygen-18 incubations showed a difference of 2 amu in the respective molecular ions, demonstrating the incorporation of molecular oxygen (Fig. 3). The fragment ions (Fig. 4) are labeled according to the cleavage of the molecule shown in the schematic inset in Fig. 3. Those fragments containing the hydroxyl oxygen at the C-18 side chain from the oxygen-18 study are 2 amu greater than those from the oxygen-16 sample. Therefore, fragments b, d, e, f, and f-a differed by 2 amu,

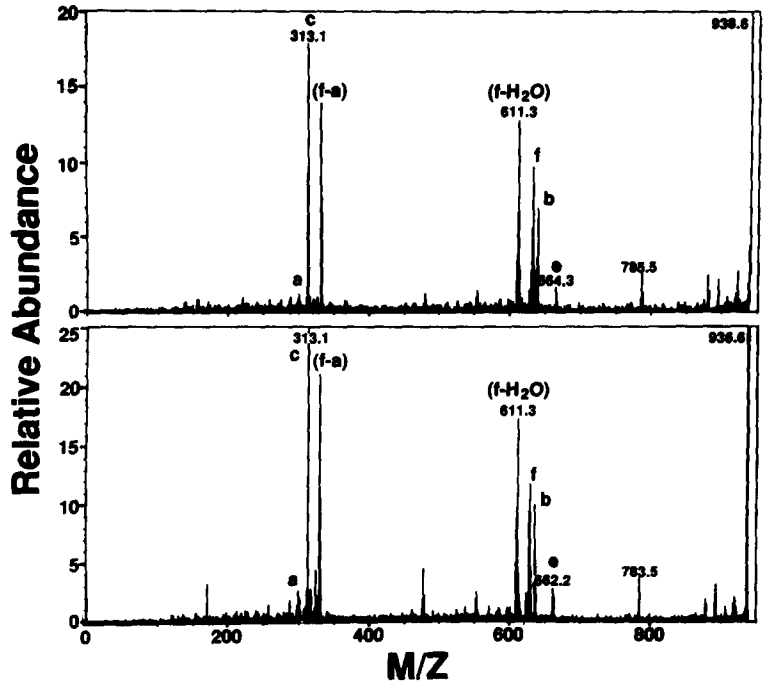
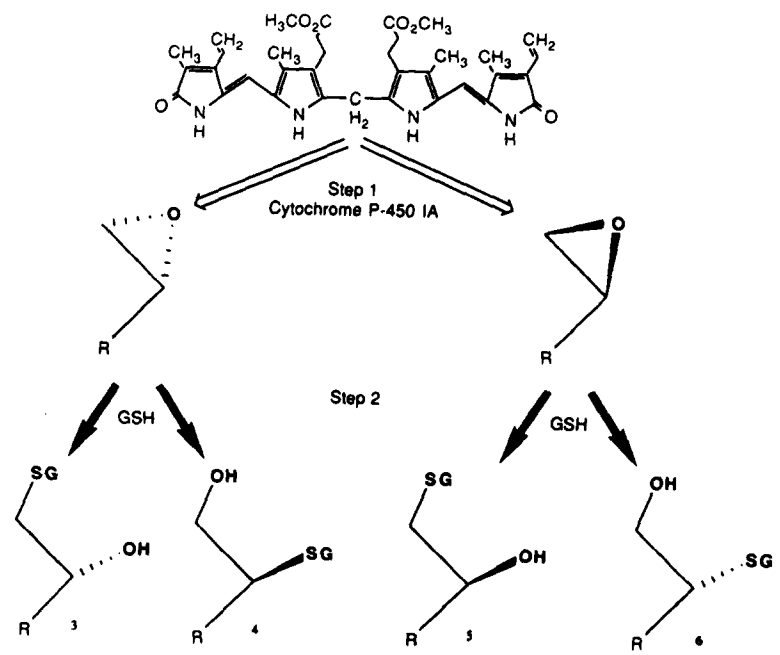


Fig. 4. Collision-activated decomposition spectra of MS-2, following mass selection of the precursor beam by MS-1.



Scheme 1. Schematic depicting the cytochrome P450-dependent oxidation of DMB during microsomal incubations and subsequent reactions with glutathione. The stereospecificity of pigment production is partially due to the oxidation of the prochiral exovinyl group, leading to the formation of two possible enantiomeric epoxide products, which differ as to which side of the vinyl group the oxygen is added. Cytochromes P450 IA are responsible for step 1 and glutathione *S*-transferase-mediated glutathione conjugation is responsible for step 2. If step 2 were to proceed non-enzymatically, then one would expect to see equal amounts of products 3 and 4 or 5 and 6 (as observed with purified P450c). R = tetrapyrrole chain minus the exovinyl group.

whereas a, c, and f-H_2O, not containing the incorporated oxygen, had identical masses.

Lack of cytosolic requirement. To determine if a cytosolic component is involved in the formation of the two DMB–glutathione conjugates, we compared conjugate formation in the presence and absence of cytosol. Table 4 illustrates that washing the microsomal pellet once or twice had no significant effect on the formation of pigments 1 and 2, but did, however, cause a 5-fold decrease in glutathione *S*-transferase activity (Table 5). These results are further confirmed in Table 6, showing that there was no significant difference in pigment formation by the microsomal fraction in the presence or absence of the cytosolic fraction. If the cytosolic glutathione *S*-transferases were involved in the formation of pigments 1 and 2, one would expect the absence of cytosol (or washing of the microsomal pellet) to show a decreased rate of conjugate formation in the reaction. Such results imply that the conjugation of glutathione with DMB may not be catalyzed by the cytosolic glutathione *S*-transferases.

DISCUSSION

Initial identification of the *in vitro* formed pigments demonstrated identical elution times (Fig. 1) and UV spectra with those pigments found *in vivo*. Incubation of the pigments with γ-glutamyltranspeptidase increased their elution times both as their tetrapyrroles (data not shown) and as their dipyrrolic azosulfanilates by reversed-phase HPLC, indicative of a less polar derivative (Fig. 2). The diazosulfanilate derivatization indicated that the conjugation occurs on the exovinyl half of the tetrapyrrole. These results were in agreement with those obtained with the pigments formed *in vivo* [13, 14].

The present study also demonstrated that the metabolism of DMB to hydroxylated DMB–glutathione conjugates by rat liver microsomes is dependent on NADPH, molecular oxygen, and glutathione (Table 1). The formation of these glutathione conjugates was inhibited by the cytochrome P450 inhibitors [20] metyrapone, 1-benzylimidazole, and α-naphthoflavone. These results suggested that cytochrome P450 mediates the formation of the DMB–glutathione conjugates. As α-naphthoflavone is a known cytochrome P448 inhibitor [20], these specific isozymes are therefore responsible. This hypothesis was further supported by studying the effects of different cytochrome P450 inducers on conjugate formation. Microsomes of Gunn rats pretreated with phenobarbital had no significant effect on DMB–glutathione formation. The large variation raised the possibility of whether the microsomal glutathione *S*-transferase was induced by phenobarbital. However, the current literature on this topic is divided [21–23]. Pretreatment with TCDD showed a 16-fold increase in the formation of pigments 1 and 2. These results implicate involvement of the cytochrome P450 IA gene family [24].

Cation FAB/MS of the pigments has shown the molecular ion to be 937 amu. A fragment ion at 307 amu was indicative of glutathione and another at 629 amu suggested a hydroxylated derivative of DMB. This was confirmed by FAB/MS/MS of the pigments after *in vitro* incubations under oxygen-16 and oxygen-18 atmospheres. An increase in the molecular ion from 937 to 939 amu was observed when the *in vitro* conjugate was formed in oxygen-18. No change was observed in the glutathione fragment at 307 amu. However the fragment at 629 amu remained as such for the oxygen-16 incubation but increased to 631 amu for the oxygen-18. This clearly shows incorporation of molecular oxygen to form the DMB–glutathione conjugates. To rule out a peroxidative mechanism as being responsible for the formation of the pigments, *in vitro* incubations were done in the presence of superoxide dismutase and/or catalase. As Table 2 shows, the antioxidant enzymes were not effective at inhibiting conjugate formation.

To determine the potential role of the cytosolic fraction upon glutathione conjugate formation, microsomes were washed twice in order to eliminate any trapped cytosolic components, such as the glutathione *S*-transferases, from the microsomal pellet. The results of Tables 4 and 5 indicate that pigment formation does not decrease with washings, despite a 5-fold decrease in glutathione conjugation of CDNB in the first wash. The slight increase in pigment formation seen with the washings in Table 4 is most likely due to ridding of non-essential protein from the microsomal pellet rather than increased enzyme activity. The residual glutathione *S*-transferase activity towards CDNB seen in the two washes of Table 5 may either represent cytosolic glutathione *S*-transferases still present in the microsomal pellet [25] and/or microsomal glutathione *S*-transferase activity. These possibilities are currently being studied. Furthermore, addition of cytosol to twice washed microsomal pellets showed no significant difference from that of controls (Table 6). These results suggest three possibilities: (1) enough cytosolic glutathione *S*-transferase activity exists within the microsomal pellet to allow glutathione conjugation to occur, so any additional glutathione *S*-transferases added show no effect; (2) the microsomal glutathione *S*-transferase is responsible for glutathione conjugate formation; or (3) glutathione conjugate formation is non-enzymatic.

Preliminary evidence (data not shown) with purified cytochrome P450c rules out the third possibility. Incubations with the purified enzyme and glutathione result in the formation of the two glutathione conjugates in a 1:1 ratio. However, *in vivo* and *in vitro* incubations result in a 7:3 ratio of glutathione conjugates (pigment 1:pigment 2). The stereospecificity of the cytochrome P450 IA gene family [26] and the proposed model of the cytochrome P450c active site by Jerina *et al.* [27] dictate that the oxidation of the prochiral exovinyl group will lead to one of two possible enantiomeric arene oxide products, differing as to which side of the DMB the oxygen is added. Therefore, enzyme-catalyzed glutathione conjugation appears to be responsible for the difference between pigments 1 and 2 (Scheme 1). Verification for the pathway which occurs can be determined by NMR analysis of the two pigments,

and is currently underway. We have evidence for the glutathione conjugation of bilirubin *in vivo* and *in vitro* (manuscript in preparation). The presence of an inducible pathway for the conjugation of bilirubin not involving the UDP-glucuronosyltransferases, as has been suggested by Kapitulnik *et al.* [28], may prove beneficial to those patients exhibiting unconjugated hyperbilirubinemia that are not responding to current medical protocol.

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