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FORMATION OF GLUTATHIONE CONJUGATES DURING OXIDATION OF EUGENOL BY MICROSOMAL FRACTIONS OF RAT LIVER AND LUNG

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Abstract—Rat hepatic and pulmonary microsomes catalyzed the formation of at least three distinct glutathione conjugates with eugenol (4-allyl-2-methoxyphenol). These three conjugates were identical with the products obtained from the chemical reaction of synthetic eugenol quinone methide and glutathione. The microsomal reaction was dependent on NADPH and oxygen and was inhibited by cytochrome P450 inhibitors such as metyrapone, 2-diethylaminoethyl-2,2′-diphenylvalerate (SKF 525-A), α-naphthoflavone and piperonyl butoxide. The enzyme responsible for eugenol oxidation was inducible with 3-methylcholanthrene but not phenobarbital pretreatment. The rate of formation of conjugates was not affected by the presence of glutathione-depleted cytosol which contained active glutathione transferase, even at low glutathione concentrations, suggesting that conjugation occurs nonenzymatically with an electrophilic metabolite of eugenol. Covalent binding to microsomal protein was observed using [^{3}H]eugenol. Cumene hydroperoxide catalyzed the formation of these same glutathione conjugates via the formation of a quinone methide-like intermediate which was detected by spectroscopic means. Our results suggest that eugenol is oxidized by cytochrome P450 to a reactive quinone methide intermediate which can then covalently modify protein or conjugate with glutathione.

Allylbenzenes occur naturally in plants and their essential oils and are used often as flavoring agents in foods and pharmaceutical preparations. Some allylbenzene derivatives, such as safrole, estragole and methyleugenol, are toxic or carcinogenic in rodents [1, 2]. The carcinogenicity of these compounds is dependent on their metabolism to electrophilic intermediates, in particular the 1′-hydroxy metabolites and, perhaps to a lesser extent, the 2′,3′-epoxides [2–4]. In contrast, eugenol (4-allyl-2-methoxyphenol), a principal ingredient of oil of clove, is considered to be nonmutagenic and noncarcinogenic and is generally recognized as safe (GRAS) by the Food and Drug Administration [5, 6]. The metabolism of eugenol in the rat involves conjugation of the free hydroxyl group with glucuronic acid and sulfate and alkene bond reduction rather than oxidation of the side chains to the presumed proximate carcinogenic metabolites [7].

Humans are exposed to eugenol through its use in dentistry as an analgesic and temporary wound dressing, in clove cigarettes, and through its presence in foods and spices [5, 8]. Eugenol exerts a number of toxic effects on cells including polymorphonuclear leukocytes, fibroblasts and dental pulp tissue, and it inhibits cellular and mitochondrial respiration [9–15]. In addition, eugenol appears to have an acute toxic effect on pulmonary cells following intratracheal administration [16]. We have studied the formation of electrophilic intermediates from eugenol by peroxidases and microsomal enzymes since such reactive metabolites may be involved in the cellular toxicity of this compound. In a previous report, we observed that peroxidase enzymes catalyze the formation of a phenoxy radical and a quinone methide-like metabolite which are capable of eliciting cytotoxicity in isolated cells [15]. The possible formation of a quinone methide is of particular interest since the formation of similar quinonoid derivatives is associated with the toxicity of other xenobiotics including acetaminophen, ellipticine, butylated hydroxytoluene and 3-methylindole [17–20]. The present study concerns the metabolism of eugenol by rat liver and lung microsomes to reactive intermediates which can be trapped with glutathione. Our results suggest that eugenol is activated by cytochrome P450 in the presence of either NADPH or cumene hydroperoxide to a quinone methide metabolite which forms at least three distinct conjugates with glutathione.

METHODS

Materials. The following biochemicals were obtained from the Sigma Chemical Co. (St Louis, MO): eugenol, glutathione (GSH), $NADP^+$, magnesium chloride, isocitrate, isocitrate dehydrogenase, sodium azide, silver(I) oxide (Ag_2O) and 1-chloro-2,4-dinitrobenzene (CDNB). Cumene hydroperoxide, piperonyl butoxide, 3-methylcholanthrene, phenobarbital, and α-naphthoflavone were purchased from Aldrich

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(Milwaukee, WI). SKF 525-A (2-diethylaminoethyl-2,2′-diphenylvalerate) was a gift of Smith, Kline & French Laboratories (King of Prussia, PA). [^{3}H]Eugenol (labeled on the methoxy group) was synthesized according to Weinberg *et al.* [21] except that ^{3}H-labeled methyl iodide (Amersham, Bucks, UK) was used instead of ^{14}C-labeled methyl iodide. The final product was purified using a semi-preparative HPLC column (Hibar LiChrosorb RP-18, 10 × 250 mm, particle size 7 μm; Merck, Darmstadt, F.R.G.) and concentrated using Sep-Pak C_{18} cartridges (Waters, Milford, MA). The radiolabeled compound had a specific activity of 3.4 μCi/μmol, and its purity was greater than 99% based on reverse phase HPLC. [^{3}H]Glutathione (100 μCi/μmol) was obtained from New England Nuclear (Boston, MA).

Synthesis of eugenol–glutathione conjugates. The eugenol–glutathione conjugates were synthesized via the reaction of eugenol quinone methide with glutathione. Eugenol quinone methide was prepared according to Zanarotti [22]. Eugenol (1.5 mmol; 0.25 g) was added to 40 mL carbon tetrachloride. Silver(I) oxide (6.5 mmol; 1.5 g) was added, and the mixture was stirred vigorously at 65° for 10 min. The reaction was filtered and allowed to cool. Glutathione (1.5 mmol; 0.46 g in 40 mL of 0.1 M phosphate, pH 8) was added to the filtrate (quinone methide), and the mixture was stirred at 37° for 3 hr. Water (50 mL) was added and the mixture decanted into a separatory funnel. The aqueous layer was washed twice with 50 mL carbon tetrachloride. The aqueous layer was frozen and lyophilized. The residue was dissolved in 1–2 mL water, and the eugenol–glutathione conjugates were collected and purified by HPLC, first with the semipreparative column, and second with repeated chromatography on an analytical column (Hibar LiChrosorb RP-18 column, 4 × 250 mm, particle size 10 μm). The samples were then analyzed by mass spectrometry and NMR.

Fast atom bombardment mass spectrometry (FABMS). FABMS was carried out on a VG 7070E double-focusing mass spectrometer with an FAB ion source, an Ion Tech atom gun and a VG 11-250 data system (VG Analytical, Manchester, UK). An aliquot of the sample, dissolved in 5 μL methanol, was applied under a slight stream of nitrogen to the FAB target already covered with the glycerol matrix. The sample was bombarded with 8 keV xenon atoms. Spectra of negative and positive ions were recorded in the range m/z 800–80 at a scan rate of 10 sec/decade and a resolution of 1000 (5% valley).

Nuclear magnetic resonance (NMR) spectrometry. All proton NMR measurements were recorded on a JEOL GSX 270 spectrometer. Assignments were made using the two-dimensional correlated spectroscopy (COSY) technique at 25°. Chemical shift values are given in parts per million (ppm) relative to tetramethylsilane (TMS $\delta_H = 0.00$). Prior to the NMR measurements, the samples were lyophilized twice in $C^2H_3O^2H$ and then dissolved in the same solvent without any pH adjustment.

Preparation of microsomes. Microsomes were prepared from male Sprague–Dawley rats (*ca.* 200 g). Animals were pretreated i.p. with corn oil (control, 3 mL of corn oil injected once), phenobarbital (80 mg/kg; 0.4 mL of 40 mg/mL solution in saline, injected once per day for 3 days), or 3-methylcholanthrene (50 mg/kg; 2 mL of 5 mg/mL solution in corn oil, injected once). Homogenates (25%) of liver and lung tissues were made in 0.25 M sucrose and centrifuged at 10,000 *g* for 20 min. The supernatant fractions were centrifuged at 100,000 *g* for 60 min. The pellets were resuspended in 0.15 M potassium chloride and recentrifuged at 100,000 *g* for 30 min. The final pellet was dissolved in 0.25 M sucrose, protein content was measured [23], and the microsomes were stored at −80°. GSH-depleted cytosol (treated with iodoacetamide and dialyzed) was a gift of Dr Bengt Jernström, Karolinska Institutet.

Incubation conditions. The standard incubation conditions consisted of 1 mg/mL microsomal protein, 1 mM eugenol, 5 mM glutathione and an NADPH-regenerating system consisting of 5 mM magnesium chloride, 5 mM isocitrate, 10 μL (0.2 units) isocitrate dehydrogenase, and 1 mM $NADP^+$ in a total volume of 1 mL of 0.1 M Tris buffer, pH 7.5. The reaction mixture was preincubated at 37° for 5 min prior to the addition of eugenol to start the reaction. Reactions were stopped by the addition of 50 μL of 100% trichloroacetic acid and centrifuged for 5 min at 12,000 rpm to pellet the protein. Samples (200 μL) were then analyzed for conjugate formation by HPLC.

HPLC analysis of eugenol–glutathione conjugates. The HPLC system consisted of Waters 501 and 510 pumps, a WISP 710B automatic injector, a Waters 740 data module and a Spectra-Physics model 770 variable wavelength detector set at 280 nm. Initially, samples were analyzed using a 10 μm, Hibar C-18 reverse phase column (Merck). Better separation was subsequently achieved using a 5 μm, Ultrasphere ODS column (Beckman), and the HPLC tracings shown in Fig. 1 were obtained with this column. The mobile phase employed was a methanol gradient. Buffer A consisted of 0.25% perchloric acid and 0.25% acetic acid adjusted to pH 3.50 with 10 M sodium hydroxide. Buffer B was 100% methanol. The initial conditions consisted of 80% A and 20% B (isocratic) from 0 to 20 min. From 20 to 35 min a linear gradient was used to change from 80% A to 50% A (curve 6). Fifty percent A was maintained until 50 min when the initial conditions were restored. The flow rate was 2 mL/min throughout the entire run.

Covalent binding to protein. The covalent binding of reactive intermediates from [^{3}H]eugenol to liver and lung microsomal protein was measured in incubations containing 0.5 μCi [^{3}H]eugenol (1 mM eugenol) under the standard reaction conditions described earlier. In some instances, glutathione was omitted from the reactions. Reactions with boiled microsomes served as background values. The reactions were stopped with the addition of 4 mL methanol and centrifuged at 1500 *g* for 3 min to pellet the protein. The pellet was then washed five to eight times with 3 mL of methanol to remove all the unbound compound. The pellet was dissolved in 1 M sodium hydroxide with heating at 60° for 60 min, the pH adjusted to 7.5, one aliquot (0.5 mL) counted for radioactivity, and another aliquot (50 μL) used for protein determination.

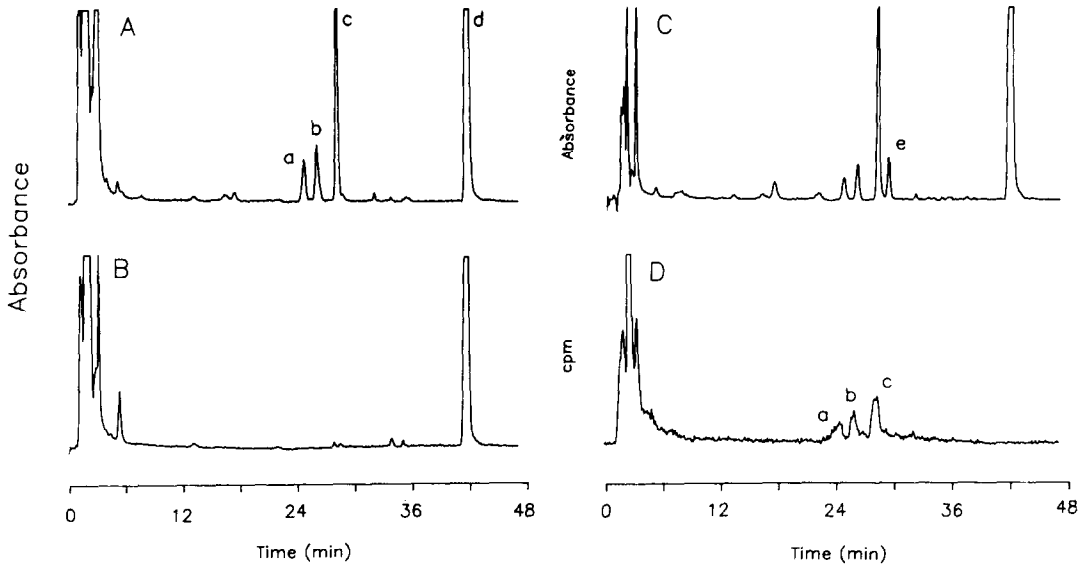


Fig. 1. HPLC analysis of products formed during the NADPH-dependent microsomal incubation of eugenol with glutathione. Reaction conditions were the same as described in Table 2. HPLC conditions are described in Methods. Panel A: complete system; B: complete system except that glutathione was omitted; C: 0.5 mM cumene hydroperoxide instead of NADPH-regenerating system was used as substrate; D: same as A except that 0.5 μCi [^{3}H]glutathione was added. Absorbance changes were measured at 280 nm. Peak identification (retention time in min): a (24.5), b (26), and c (28) are eugenol–glutathione conjugates; d (44) is parent eugenol; and e (30) is an additional unidentified metabolite formed only in the presence of cumene hydroperoxide.

Assay for eugenol quinone methide formation. Formation of eugenol quinone methide was followed on an Aminco DW-2 spectrophotometer operating in double beam mode by monitoring the absorbance change at 350 nm. The reference beam (mono 2) was set at 450 nm. Incubation conditions consisted of 0.5 mg/mL of 3-methylcholanthrene-induced liver microsomes, 0.5 mM eugenol, 0.5 mM cumene hydroperoxide and 1 mM glutathione in 1 mL of 0.1 M Tris buffer, pH 7.5. The reactants were preincubated at 37° for 2 min before the addition of eugenol to start the reaction.

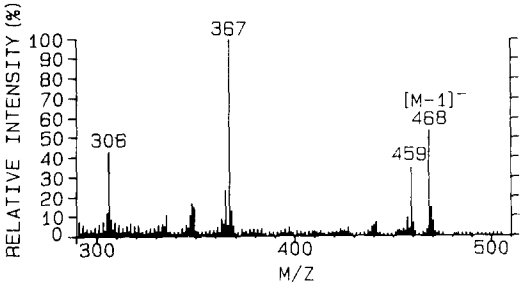


Fig. 2. FAB mass spectrum of eugenol–glutathione conjugate (metabolite a) using glycerol as matrix. The spectrum is recorded in negative-ion mode. Ions at m/z 367 and 459 originate from the matrix.

RESULTS

Identification of eugenol–glutathione conjugates. The microsomal incubation of eugenol in the presence of glutathione and an NADPH-regenerating system led to the formation of three distinct metabolites (peaks a, b and c, Fig. 1A). The formation of these products was dependent on the presence of glutathione (Fig. 1B). The HPLC profile of the reaction products in the presence of [^{3}H]glutathione indicated that each of these products contained glutathione (Fig. 1D). Therefore, these metabolites appear to be eugenol–glutathione conjugates. Cumene hydroperoxide also supported the formation of the same three conjugates, with an additional product as well (peak e, Fig. 1C). The three metabolites (a, b and c) were collected and analyzed by FABMS. Figure 2 shows the negative ion spectrum obtained from metabolite (a). A quasi-molecular ion ([M-1]$^-$) of 468 daltons was obtained consistent with the molecular weight of the addition product between glutathione and eugenol quinone methide. An abundant fragment ion at m/z 306 was present indicative of a glutathione conjugate [24]. Identical spectra were also obtained from metabolites (b) and (c) (not shown). Analysis of negative ions was better than that of positive ions for these compounds, due to a much better response relative to the matrix ions.

Peaks (a) and (c) were analyzed further by NMR. The chemical shift values on protons in the gluta-

Table 1. Chemical shift values for eugenol and peaks a and c

	Eugenol		Peak a		Peak c	
Proton	1*	2	1	2	1	2
H_1	5.03	4.96	5.18	5.12	3.31	3.00
H_2	4.99	4.93	5.12	5.03	3.31	3.00
H_3	5.94	6.30	6.03	5.96	6.01	6.32
H_4	3.26	3.30	4.47	4.60	6.44	6.50

Column 1 contains experimental proton NMR values in ppm. Column 2 contains theoretical values calculated for H_1, H_2 and H_3 with R_1 as a proton and for H_4 with R_1 as a Ph, S-R as S-Me[2] [25]. See Fig. 3 for structural details.

thione moiety and the phenyl group in the conjugates were consistent (within ± 0.2 ppm, except for the C-β-$H_{1,2}$ in peak (a) which showed a downfield shift of 0.4 ppm) with measurements made with glutathione and eugenol alone (not shown). The chemical shift values for the allylic protons in eugenol and peaks (a) and (c) are given in Table 1. A comparison of the theoretical values [25] with sample values led to the proposed structures which have glutathione attached to C-1′ (peak a) or C-3′ (peak c). The structures are shown in Fig. 3. The *trans* configuration in peak (c) was suggested by a coupling constant of 16 Hz between H_3 and H_1. The location of the glutathione moiety on peak (b) could not be resolved. However, it does not appear to be located on the ring. Radioactive glutathione and eugenol were used to quantitate the amount of conjugates formed. It is apparent from Fig. 1D that the quantity of each metabolite formed is approximately equal. This finding is also consistent with the response found during the recording of the FAB spectra. Metabolite (c) appears to have a much greater UV absorbance at 280 nm, however, than do the other two metabolites (Fig. 1A). This alteration of the chromophore is expected with the proposed structure of peak (c) in which the double bond occurs between C-1′ and C-2′. For example, we have observed that isoeugenol (which has a C-1′—C-2′ double bond) has a higher absorbance at 280 nm than does eugenol.

The formation of conjugates was dependent on protein concentration (not shown). The amount of conjugates formed increased linearly with protein concentration up to 1 mg/mL. Subsequent experiments used a protein concentration of 1 mg/mL. In an incubation of 90 min, approximately 28 nmol of conjugates was formed, amounting to about 2.8% of the eugenol present at the start of the incubation. These values reflect the total amount of conjugates formed (metabolites a + b + c) as do the rest of the data shown in this report. The formation of conjugates was linear up to 30 min (not shown). Therefore, this time point was used in subsequent experiments.

Inhibition of conjugate formation. The effect of the omission of various reaction components on the formation of eugenol–glutathione conjugates is shown in Table 2. In addition to the effect of omitting glutathione which was already mentioned, omission of the NADPH-generating system, eugenol or microsomes resulted in complete lack of formation of conjugates. The substitution of heat-inactivated microsomes also inhibited formation of conjugates, indicating the enzymatic nature of this reaction. Table 3 lists the effects of several cytochrome P450 inhibitors on the formation of eugenol–glutathione conjugates. Metyrapone, SKF 525-A, piperonyl butoxide and α-naphthoflavone were effective inhibitors of conjugate formation. The reaction was also oxygen-dependent since reactions conducted under a nitrogen atmosphere prevented conjugate formation. The peroxidase inhibitor sodium azide was not effective at inhibiting conjugate formation.

Pretreatment of animals with the cytochrome P450 inducers phenobarbital or 3-methylcholanthrene demonstrated that the form of enzyme responsible for the activation of eugenol is 3-methylcholanthrene inducible (Table 4). Control liver microsomes catalyzed conjugate formation at a rate of 0.66 nmol/min/mg protein. Phenobarbital had no effect on the rate of conjugate formation, while 3-methylcholanthrene enhanced the rate of conjugate formation by 2-fold. Control lung microsomes catalyzed conjugate formation at a rate of 0.05 nmol/min/mg protein.

Formation of a reactive, electrophilic intermediate. The formation of reactive electrophilic intermediates

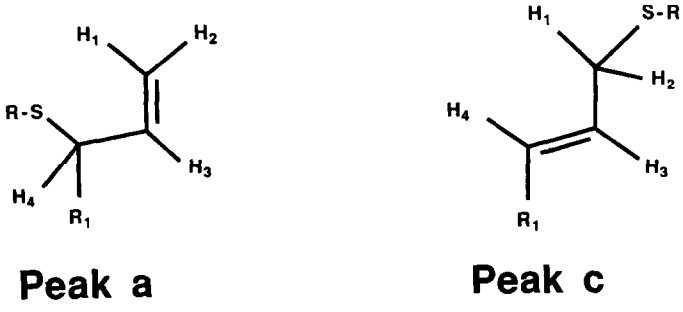


Fig. 3. Proposed structures of two eugenol–glutathione conjugates (peaks a and c). Only the binding sites on the allylic portion of the eugenol molecule are shown. R-S represents the glutathione moiety and R_1 the remaining eugenol moiety.

Table 2. Effects of omission of reaction components on the formation of eugenol–glutathione conjugates in control rat liver microsomes

Reaction	Conjugate formation (nmol/min/mg)
Complete system	0.53 ± 0.02*
−Glutathione	ND†
−NADPH-regenerating system	ND
−Eugenol	ND
−Microsomes	ND
+Boiled microsomes	ND

Complete system contained 1 mg/mL control rat liver microsomal protein, 1 mM eugenol, NADPH-regenerating system and 5 mM glutathione in a total of 1 mL of 0.1 M Tris buffer, pH 7.5. Reactions were terminated after 30 min of incubation at 37° by the addition of trichloroacetic acid (5% final concentration). Boiled microsomes were heated at 80° for 10 min prior to inclusion in the indicated incubations.

* Mean ± SE of triplicate samples.

† ND = not detected.

can be demonstrated through covalent binding of radioactive compounds to protein. Table 5 illustrates that both liver and lung microsomes are capable of activating eugenol to a covalently bound metabolite. The presence of 5 mM glutathione almost completely inhibited the covalent binding of this metabolite to protein in both tissues. These results demonstrate that eugenol was metabolized to a reactive, electrophilic intermediate which was capable of covalently modifying protein. This was further substantiated by testing the effect of glutathione-depleted cytosol on the formation of eugenol–glutathione conjugates in microsomal incubations. The effect of glutathione-depleted cytosol (which still contained an active glutathione transferase) was tested at both low (0.25 mM) and high (5 mM) glutathione concentrations (Table 6). In the presence of low concentrations of glutathione, one would expect cytosol to enhance the rate of conjugate formation if glutathione transferase was involved in the conjugation reaction. However, the presence of cytosol had no effect on the rate of conjugate formation at either low or high glutathione concentrations, implying that the conjugation of glutathione with the electrophilic metabolite of eugenol occurs nonenzymatically.

Finally, we investigated the nature of this reactive intermediate. Since the chemical reaction of eugenol quinone methide with glutathione resulted in the formation of the same three conjugates as did the microsomal incubations, we reasoned that the quinone methide metabolite may be the reactive intermediate. Eugenol quinone methide absorbs at

Table 5. Covalent binding of [^{3}H]eugenol to rat liver and lung microsomal protein

Tissue	Eugenol bound (pmol/min/mg) −GSH	Eugenol bound (pmol/min/mg) +GSH
Liver	196 ± 10	1 ± 0
Lung	78 ± 10	9 ± 0.3

Reaction conditions were as described in Table 2, with the addition of 0.5 μCi [^{3}H]eugenol and 5 mM glutathione (GSH, where indicated) to each reaction tube. Values are means ± SE of triplicate samples.

Table 3. Effects of cytochrome P450 inhibitors on the formation of eugenol–glutathione conjugates in control rat liver microsomes

Reaction	Conjugate formation* (nmol/min/mg)	% of Complete system
Complete system	0.66 ± 0.04	100
+Metyrapone	0.18 ± 0.03	27
+SKF 525-A	0.10 ± 0.01	15
+Piperonyl butoxide	0.08 ± 0.02	12
+α-Naphthoflavone	0.17 ± 0.02	26
+N_2 atmosphere	0.05 ± 0.01	8
+Azide	0.64 ± 0.04	97

Reactions conditions were the same as described in Table 2 using control rat liver microsomes. The concentration of inhibitors was 1 mM except for α-naphthoflavone (0.5 mM). For incubations with nitrogen atmosphere, the tubes were bubbled with nitrogen gas for 2 min prior to the initiation of the reaction and were sealed during the incubation.

* Values are means ± SE of triplicate samples.

Table 4. Effects of enzyme inducers on formation of eugenol–glutathione conjugates in rat liver microsomes

Tissue	Treatment	Conjugate formation (nmol/min/mg)
Liver	Control	0.66 ± 0.05
	Phenobarbital	0.57 ± 0.01
	3-Methylcholanthrene	1.34 ± 0.06
Lung	Control	0.05 ± 0.003

Reaction conditions were the same as described in Table 2 except for the source of microsomal protein. Values are means ± SE of triplicate samples.

Table 6. Effect of cytosol (GSH-depleted) on microsomal formation of eugenol–glutathione conjugates

Reaction	GSH (mM)	Conjugate formation (nmol/min/mg)
Microsomes	0.25	0.62 ± 0.04
Microsomes + cytosol	0.25	0.68 ± 0.01
Microsomes	5.0	0.64 ± 0.03
Microsomes + cytosol	5.0	0.55 ± 0.02

Reaction conditions were as described in Table 2. GSH-depleted cytosol (final concentration 1 mg/mL) was added where indicated. The activity of CDNB–glutathione transferase in the GSH-depleted cytosol was measured as 0.4 μmol/min/mg. Values are means ± SE of triplicate samples.

350 nm [15]. Therefore, it was not possible to measure the formation of this metabolite in incubations containing NADPH since these two compounds would interfere with each other. Cumene hydroperoxide does not absorb at this wavelength, however, and in incubations with 3-methylcholanthrene-induced microsomes and eugenol, an increase in absorbance at 350 nm was observed (Fig. 4). The formation of this product was prevented completely by glutathione present at the beginning of the reaction. Glutathione added after the reaction had started caused a decrease in the absorbance. Although the use of cumene hydroperoxide as a substrate implies a peroxidative method of oxidation of eugenol, the same three conjugates were formed as occurred with NADPH, suggesting that a common intermediate is being formed. These results are consistent with the hypothesis that the reactive intermediate being formed is eugenol quinone methide.

DISCUSSION

It is well established that the toxic effects of a broad spectrum of xenobiotics are mediated through reactive intermediates formed during the metabolism of the parent compound. We have studied the NADPH-dependent oxidation of eugenol by rat liver and lung microsomes and have demonstrated the formation of an electrophilic metabolite which covalently binds to protein and glutathione. Glutathione conjugates of eugenol have not been reported previously. The rate of formation of the eugenol–glutathione conjugates is comparable to the rate reported for butylated hydroxytoluene in untreated rat liver microsomes [26]. Our results with inducers and inhibitors of the cytochrome P450 monooxygenase system indicate that a 3-methylcholanthrene-inducible form of cytochrome P450 is responsible for the oxidation of eugenol in rat liver microsomes.

We further studied the nature of the reactive intermediate formed during eugenol oxidation. Conjugation of glutathione with electrophilic compounds is often assisted by the glutathione *S*-transferases, a family of enzymes present primarily in the cytosolic fractions of most tissues [27]. However, the presence of a glutathione-depleted cytosolic fraction containing active glutathione *S*-transferase had no effect on the rate of formation of eugenol–glutathione conjugates, at either low or high glutathione concentrations. This suggests that the conjugation of glutathione with the reactive metabolite of eugenol

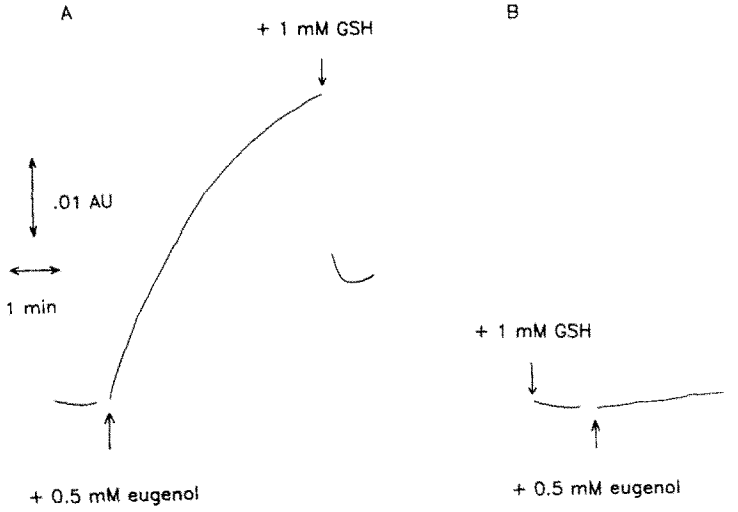
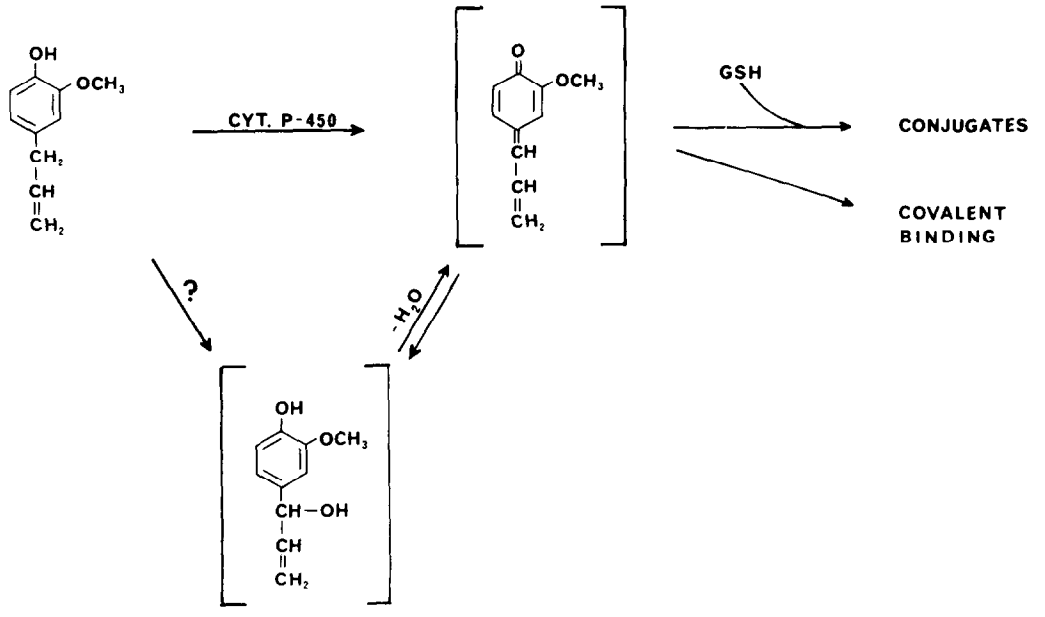


Fig. 4. Oxidation of eugenol during microsomal incubations with cumene hydroperoxide. Reactions contained 0.5 mM cumene hydroperoxide, 0.5 mM eugenol, 1 mM glutathione (where indicated), 0.5 mg/mL of 3-methylcholanthrene-induced liver microsomes in 1 mL of 0.1 M Tris buffer, pH 7.5, at 37°. Reactants were preincubated for 2 min prior to the addition of eugenol to start the reaction. Absorbance changes at 350 nm were followed over time. Panel A: reaction was started in the absence of glutathione (added at the arrow). Panel B: glutathione was present at the start of the reaction.



Scheme 1. Schematic depicting the cytochrome P450-dependent oxidation of eugenol during microsomal incubations and subsequent reactions with glutathione and protein.

proceeds nonenzymatically. The reactive metabolite of eugenol also forms covalent adducts with protein, perhaps by reacting with sulfhydryl groups (Table 5).

In addition, we observed that three different glutathione conjugates were formed with eugenol. When synthetic eugenol quinone methide was incubated with glutathione, the same three conjugates were formed. Theoretically, glutathione may add to several sites on the quinone methide, both on the allyl moiety (C-1′ and C-3′) and perhaps also on the ring (C-3 and C-5). Our NMR data indicate that glutathione conjugates form on C-1′ and C-3′. These results suggest that the reactive intermediate may be the quinone methide. Other evidence which supports the possible formation of quinone methide in these reactions includes data from the peroxidative metabolism of eugenol. The horseradish peroxidase and myeloperoxidase-dependent oxidation of eugenol lead to the formation of the same three glutathione conjugates and this is thought to occur through the formation of a quinone methide intermediate [15, 28]. Also, in Fig. 4 of the present paper, the cumene hydroperoxide-dependent oxidation of eugenol supports the formation of at least three glutathione conjugates, and this is accompanied by an increase in absorbance at 350 nm (quinone methide) which is sensitive to the presence of glutathione.

Several allylbenzene derivatives are known to form 1′-hydroxy metabolites which are thought to lead to the formation of electrophilic species which are responsible for the toxicity or carcinogenicity elicited by these compounds [2, 3]. These compounds include safrole, estragole and methyleugenol [29, 30]. In particular, the sulfate of 1′-hydroxysafrole and 1′-hydroxyestragole are thought to be the major electrophilic metabolites reacting with hepatic DNA *in vivo* [31, 32]. The formation of 1′-hydroxyeugenol has not been reported in enzymatic incubations. Eugenol may form 1′-hydroxyeugenol in microsomal incubations but our evidence indicates that the species responsible for covalent binding with glutathione is the quinone methide. The 1′-hydroxyeugenol may be formed initially in the reaction and then rearrange (through loss of water) to form the quinone methide or, alternatively, the quinone methide may be formed directly (Scheme 1).

Our evidence suggests that the metabolism of at least one allylbenzene, eugenol, proceeds through a quinone methide intermediate. This reactive metabolite may then covalently modify proteins or conjugate with glutathione. The formation of a metabolite which is capable of depleting glutathione suggests that perhaps some of the cellular toxicity elicited by eugenol is dependent on its metabolism to this reactive intermediate. This may also help explain, in part, why eugenol is not genotoxic. Safrole, estragole and methyleugenol are carcinogenic allylbenzenes whose structures prohibit the formation of a quinone methide metabolite without prior metabolism (such as O-demethylation). Eugenol has a free hydroxyl group which leads to its rapid elimination by conjugation with sulfate and glucuronic acid. In fact, eugenol is known to induce glucuronide conjugation enzymes [33, 34]. Thus, a smaller percentage of eugenol would be available for the formation of 1′-hydroxy metabolites. In addition, the formation of quinone methide would lead to conjugation with glutathione. Thus, no genotoxic metabolites would be formed. Conversely, 1′-hydroxy metabolites of safrole, estragole and methyleugenol may be formed more readily and persist to form the reactive sulfate esters which are potentially genotoxic.

The formation of electrophilic quinonoid metab-

olites from xenobiotic compounds which may play a role in toxicity, particularly from phenols, is amply supported in the literature. The quinone imine of acetaminophen is thought to be responsible for hepatotoxicity seen with large doses of this compound [19]. The quinone methide of butylated hydroxytoluene is thought to play a role in pulmonary and hepatic toxicity caused by this compound in mice and rats [18]. Quinone imines from ellipticine are thought to be involved with toxicity resulting from administration of this drug [20]. In addition, an imine methide from 3-methylindole has been suggested to play a role in pulmonary toxicity from this compound [17]. These reactions are all mediated by cytochrome P450 although peroxidases also form these two-electron oxidation products.

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