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THE SIDE-CHAIN EPOXIDATION AND HYDROXYLATION OF THE HEPATOCARCINOGENS SAFROLE AND ESTRAGOLE AND SOME RELATED COMPOUNDS BY RAT AND MOUSE LIVER MICROSOMES

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Summary

Safrole, estragole, and their 2′,3′-oxides were hydroxylated by hepatic microsomes from rats and mice at the 1′-carbon; *trans*-anethole was hydroxylated at the 3′-carbon; and safrole, estragole, and their 1′-hydroxy derivatives were epoxidized at the 2′,3′-double bond. The 2′,3′-epoxidation of eugenol was just detectable. The formation of these metabolites was dependent on an NADPH-generating system and on cytochrome P-450.

In the absence of 3,3,3-trichloropropylene oxide little or no safrole-, estragole-, or eugenol-2′,3′-oxide was recovered when these oxides were added to the incubations; recoveries of 50—70% were obtained in its presence. The recoveries of the 2′,3′-oxides of 1′-hydroxy-safrole and of 1′-hydroxyestragole were 50—80% in the absence of trichloropropylene oxide and nearly quantitative in its presence. All incubations for analysis of epoxidation rates contained trichloropropylene oxide.

The rates of metabolite formation ranged from about 0.4 nmol of eugenol-2′,3′-oxide/mg protein/h by female rat liver microsomes to about 270 nmol of *trans*-3′-hydroxyanethole/mg protein/h for male rat liver microsomes. The rates of epoxidation and hydroxylation were greater for the estragole derivatives than for the safrole derivatives. The rates of epoxidation of safrole and estragole were greater than for their 1′-hydroxy derivatives.

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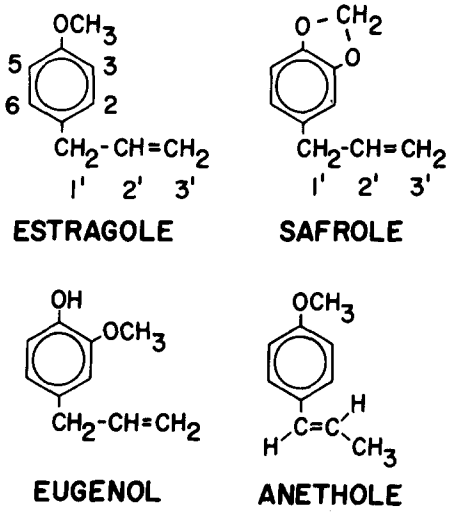

Fig. 1. The structural formulae of the naturally occurring alkenylbenzenes discussed in the text.

Introduction

Safrole (1-allyl-3,4-methylenedioxybenzene) and estragole (1-allyl-4-methoxybenzene) (Fig. 1) are weak hepatocarcinogens [1—6] that occur in certain spices and essential oils used as flavoring agents [7,8]. Earlier studies from this laboratory showed the in vivo metabolism of these two carcinogens to their 1′-hydroxy derivatives [6,9] and the stronger hepatocarcinogenicity of the 1′-hydroxy derivatives as compared to the activities of the parent compounds [5,6,10]. Wislocki et al. [11] further showed the NADPH-dependent metabolism of 1′-hydroxysafrole to its 2′,3′-oxide by rat and mouse liver microsomes and the 3′-phosphoadenosine-5′-phosphosulfate-dependent metabolism of 1′-hydroxysafrole to another electrophilic derivative, presumably its sulfuric acid ester. The epoxidation of safrole, as evidenced by the formation of the 2′,3′-dihydrodiol, either in vivo or in rat liver cells in culture, has also been demonstrated [12,13]. Similar evidence has been presented for the epoxidation by cultured liver cells of eugenol (1-allyl-4-hydroxy-3-methoxybenzene) [14], another spice component [7,8].

Estragole, safrole, eugenol, *trans*-anethole (4-methoxy-1-propenylbenzene, a common spice flavor), and their side-chain hydroxy derivatives showed no or only weak direct mutagenic activity for *S. typhimurium* TA 100 [10,14—18]. The 2′,3′-oxides, on the other hand, showed relatively strong dose-dependent mutagenic activities in the absence of an added activating system [10,15,16]. Thus, the present quantitative study of the capacities of mouse and rat liver microsomes for the side-chain hydroxylation and epoxidation of the above-mentioned compounds and their epoxy or hydroxy derivatives was undertaken as part of an investigation of the possible roles of these metabolites in the carcinogenic activities of these naturally occurring allylic and propenylic benzenes.

Materials and Methods

Instrumentation and chromatography

Ultraviolet spectra were measured with a series EU-700 double beam spectrophotometer equipped with a Model EU-205-11 strip chart recorder (GCA/McPherson Instruments, Acton, MA). Infrared spectra were recorded with a Beckman IR-10 spectrometer. Melting points were determined visually with a Fisher-Johns apparatus.

Preparative column chromatography utilized silica gel 60, 0.063—0.2 mm (Merck, Darmstadt, F.R.G.). The column eluates were monitored by thin-layer chromatography (TLC) on 0.25-mm silica gel plates (Brinkman Instruments, Westbury, NY, or Merck) which were developed with *n*-hexane/diethyl ether or chloroform/methanol mixtures and viewed under a 254 nm mercury lamp. For further detection of small amounts of alkenylbenzene derivatives and impurities, the silica plates were sprayed with a vanillin/sulfuric acid reagent [19] and heated at 100—120°C for about 5 min.

All materials used for high performance liquid chromatography (HPLC) were products of Waters Associates, Milford, MA. HPLC was performed on a Model 201 liquid chromatograph equipped with a Model U6K injector system, a Model 660 solvent programmer, and a Model 440 ultraviolet detector. This chromatography utilized either a microparticulate reverse-phase octadecylsilane column (μBondapak C18) eluted with acetonitrile-water or methanol-water mixtures or a microparticulate silica column (μPorasil) eluted with *n*-hexane/diethyl ether mixtures.

Chemicals

trans-Anethole, estragole, safrole, and eugenol were obtained from Aldrich Chemical Co. (Milwaukee, WI). 1′-Hydroxyestragole, 1′-hydroxysafrole, *trans*-3′-hydroxyanethole, 1′-hydroxysafrole-2′,3′-oxide, safrole-2′,3′-oxide, 1′-hydroxyestragole-2′,3′-oxide, estragole-2′,3′-oxide, and eugenol-2′,3′-oxide were synthesized and purified as described in previous papers from this laboratory [6,9,10,15]. Column chromatography on silica gel with the indicated *n*-hexane/diethyl ether (v:v) mixtures was used for purification of anethole (5:1), estragole (5:1), safrole (2:1), eugenol (1:2), 1′-hydroxysafrole (2:1), 1′hydroxyestragole (2:1), safrole-2′,3′-oxide (1:2), and estragole-2′,3′-oxide (1:2). All compounds were stored at —20°C, and their purities were monitored periodically. The purity of each compound was estimated by HPLC to be greater than 99%.

Safrole-2′,3′-dihydrodiol and the new compound estragole-2′,3′-dihydrodiol were synthesized by acid hydrolysis of the corresponding 2′,3′-oxides according to the general procedure of Watabe and Akamatsu [20], except that 10 ml of water and 2.5 ml of 95% ethanol per g of epoxide were included in the initial reaction mixtures. The reactions were allowed to proceed in the dark for 2 h at 60°C with vigorous stirring. The diol-containing diethyl ether extracts were dried over anhydrous $MgSO_4$ and Na_2CO_3, and the ether was removed under reduced pressure.

The solid residue containing estragole-2′,3′-dihydrodiol was dissolved in chloroform-methanol (20:1, v:v) and chromatographed on a column of silica

gel with the same solvent mixture. The fractions containing only estragole-2′,3′-dihydrodiol (as determined by TLC) were pooled, and the solvent was evaporated to yield crystals of m.p. 40°C. Ultraviolet: λ_{max}, 225 nm (ϵ = 10 400), 277 nm (ϵ = 1690), 284 nm (ϵ = 1430) in methanol. Infrared spectrum (absorption maxima in cm^{-1}; s, strong; m, moderate; w, weak) in Nujol (corrected for the Nujol control): 3380(s, broad), 1610 (m), 1580 (w), 1505 (m), 1295 (w), 1240 (s), 1170 (m), 1065 (m), 1020 (s), 800 (m), 530 (broad, w). Additional, although ill-defined, bands at 2890 (s) and 1440 (s) were seen in the spectrum of the neat dihydrodiol. Analyses for the constituent elements (Huffman Laboratories, Inc., Wheatridge, CO) were all within 0.3% of theory.

The crude product containing safrole-2′,3′-dihydrodiol was dissolved in diethyl ether at 25°C, and the diol was crystallized from the solution at 0°C. The crystals were dissolved in chloroform-methanol (20:1, v:v) and chromatographed as described above for estragole-2′,3′-dihydrodiol. Evaporation of the solvent yielded white crystals of m.p. 78—79°C (79—80°C [20]). The ultraviolet and infrared spectra of this compound were essentially identical with those previously reported [20].

The synthetic hydroxy and epoxy derivatives used for these studies were presumably racemates.

In vitro metabolism of the alkenylbenzenes and their derivatives

Young adult rats (Fischer) and mice (CD-1) from the Charles River Breeding Laboratory, Wilmington, MA were housed in screen-bottomed cages and fed Wayne Breeder Blox (Allied Mills, Inc., Chicago, IL). The animals were fasted for 12—15 h before they were killed by decapitation.

The livers were finely minced and 20% homogenates (w/v) were prepared in 0.25 M sucrose buffered with 5 mM Tris-HCl (pH 8.0 at 5°C) by 10—12 strokes of a Teflon homogenizer operated at about 2000 revs. per min in a glass tube (Tri R Laboratory Instruments, Rockville Centre, NY). The supernatant fraction obtained by centrifugation of the homogenate at 13 000 × *g* for 10 min was then centrifuged at 40 000 × *g* for 40 min. The microsome-containing pellet was resuspended in 0.25 M sucrose buffered with Tris-HCl and the suspension was centrifuged at 43 000 × *g* for 60 min. The microsomes in the pellet were resuspended in the Tris-buffered sucrose. The microsome isolation was performed at 5°C.

The reactions were carried out in a 5-ml volume that contained 2.0 mM alkenylbenzene (added in 0.1 ml of ethanol)/5.0 mM $MgCl_2$/1.0 mM EDTA/0.5 mM NADP/5.0 mM glucose-6-phosphate/10 u of glucose-6-phosphate dehydrogenase (Worthington Biochemical Corp., Freehold, NJ)/100 mM bis-(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane buffer (pH 7.4), and a freshly prepared microsome suspension containing 7—12 mg of protein (according to the method of Lowry et al. [21]). 3,3,3-Trichloropropylene oxide in 0.05 ml of ethanol was added at an 8.0 mM final concentration when safrole-2′,3′-oxide or estragole-2′,3′-oxide was the substrate; in all other cases trichloropropylene oxide was added at a final concentration of 4.0 mM. If only 4 mM trichloropropylene oxide was used, occasional interference in determination of 1′-hydroxysafrole- and 1′-hydroxyestragole-2′,3′-oxide from safrole- and estragole-2′,3′-oxide was encountered; the dihydrodiols formed by hydrolysis of the

two latter epoxides eluted very close to the 1′-hydroxy-2′,3′-oxides. In addition, each run included control incubations (the same reaction mixture without any alkenylbenzene) and recovery incubations in which the expected side-chain hydroxy or epoxy derivatives at low levels (within the ranges of or somewhat above the amounts expected from the substrate), were incubated individually with the complete reaction mixture, but in the absence of any other substrate. All incubations were for 20 min. Additional experiments showed that under these conditions each of the oxidation reactions proceeded essentially linearly for at least 20 min and with up to at least 12 mg of microsomal protein.

The reaction mixtures were incubated in 50-ml Erlenmeyer flasks at 37°C with shaking in air. The reactions were terminated by rapid cooling on ice, which was followed immediately by vigorous shaking with 5 ml of diethyl ether. After 2 additional extractions with 10 ml of diethyl ether, the combined ether extracts were dried with Na_2CO_3 and $MgSO_4$, evaporated to dryness under reduced pressure, and dissolved in acetonitrile/water or methanol/water mixtures for HPLC on a μBondapak C18 reverse-phase column which was eluted with the same solvent mixtures. The retention times and the peak-heights, as compared to those of synthetic standards, were used for the identification and quantitation of the metabolites. For confirmation, synthetic standards were added to extracts of the incubation mixtures to verify that the retention times were identical by co-chromatography with two or more chromatographic systems. The chromatographic columns and the usual solvents, flow rates, and retention times for the various metabolic products were: *trans*-3′-hydroxyanethole (μBondapak C18, 31% acetonitrile, 2.0 ml/min, 8 min; μPorasil, 50% diethyl ether in *n*-hexane, 2.0 ml/min, 8 min); 1′-hydroxysafrole (μBondapak C18, 31% acetonitrile, 2.0 ml/min, 6.5 min; μPorasil, 14% diethyl ether in *n*-hexane, 2.0 ml/min, 17 min); safrole-2′,3′-oxide (μBondapak C18, 31% acetonitrile, 2.0 ml/min, 11.5 min; μPorasil, 14% diethyl ether in *n*-hexane, 2.0 ml/min, 5 min); 1′-hydroxyestragole (μBondapak C18, 33% acetonitrile, 2.0 ml/min, 6 min; μPorasil, 25% diethyl ether in *n*-hexane, 2.0 ml/min, 6.5 min); estragole-2′,3′-oxide (μBondapak C18, 33% acetonitrile, 2.0 ml/min, 10 min; μPorasil, 12% diethyl ether in *n*-hexane, 2.0 ml/min, 5 min); 1-hydroxysafrole-2′,3′-oxide (μBondapak C18, 14% acetonitrile, 2.5 ml/min, 8 min; μBondapak C18, 25% methanol, 2.0 ml/min, 10.5 min; μPorasil, 40% diethyl ether in *n*-hexane, 2.0 ml/min, two peaks presumably diastereomers, 9.5 min and 12 min); 1′-hydroxyestragole-2′,3′-oxide (μBondapak C18, 14% acetonitrile, 2.5 ml/min, 8 min; μBondapak C18, 25% methanol, 2.0 ml/min, 11 min; μPorasil, 40% diethyl ether in *n*-hexane, 2.0 ml/min, two peaks presumably diastereomers, 11.5 min and 14 min); eugenol-2′,3′-oxide (μBondapak C18, 20% acetonitrile, 2.1 ml/min, 7 min; μBondapak C18, 30% methanol, 2.6 ml/min, 7.5 min; safrole-2′,3′-dihydrodiol (μBondapak C18, 14% acetonitrile, 2.5 ml/min, 7 min); estragole-2′,3′-dihydrodiol (μBondapak C18, 14% acetonitrile, 2.5 ml/min, 7.5 min). The acetonitrile and methanol were diluted with water. Column void volumes were approx. 3 ml. The elution of anethole derivatives was monitored at 254 nm, while the elution of the metabolites of eugenol, estragole, and safrole derivatives was monitored at 280 nm.

Assay for electrophilic reactivity

[U-^{14}C]Guanosine (0.6 mM; ICN Pharmaceuticals, Inc., purified by chromatography on cellulose thin-layers and diluted to a specific activity of about 0.1 mCi/mmol) was incubated with 47 mM 2′,3′-oxide at 37°C in 1:1 acetone: 0.05 M sodium phosphate buffer (pH 7.0). After 5, 25, and 97 h, 50-μl aliquots of the reaction mixture were chromatographed as a 2.5 cm band on plastic-backed cellulose thin-layer plates (Brinkmann Instruments, Inc., Westbury, NY) with ethyl acetate:acetic acid:water (70:20:10). The chromatograms were dried and developed a second time with the same solvent mixture. This solvent system afforded sharper bands than did the system previously used by Wislocki et al. [11]. Each chromatogram was divided into 6—8 sections, and the cellulose from each section was transferred to Scintisol (Isolab, Inc., Akron, OH). The percent reaction was defined as the percentage of ^{14}C that moved with an R_f higher than that of unreacted guanosine, after subtraction of blanks (^{14}C from the analogous regions of chromatograms of incubation mixtures that contained no 2′,3′-oxide). The blanks were generally less than 1% of the ^{14}C applied to the plate.

After 97 h at 37°C, the reaction products were separated by HPLC on a μBondapak C18 reverse-phase column. The column was eluted first with a 20-min, slightly concave gradient (curve no. 7, Model 660 solvent programmer, Waters Associates) of 5—30% or 0—35% acetonitrile in water and then isocratically with 30% or 35% acetonitrile; the flow rates were 2.0 ml/min. The column effluent was collected in 30-s fractions, which were assayed for ^{14}C by scintillation spectroscopy after addition of Scintisol.

Results

Microsomal side-chain hydroxylation and epoxidation

Safrole was metabolized by hepatic microsomes from male and female rats and mice to safrole-2′,3′-oxide and to 1′-hydroxysafrole, and each of these metabolites was converted to 1′-hydroxysafrole-2′,3′-oxide (Table I). Similarly, estragole was oxidized by hepatic microsomes at the 2′,3′- double bond and was hydroxylated at the 1′-position, and each of these metabolites was converted to 1′-hydroxyestragole-2′,3′-oxide. In general, the rates of epoxidation of the 1′-hydroxy compounds (5—18 and 2—10 nmol/mg protein/h for 1′-hydroxyestragole and 1′-hydroxysafrole, respectively) were less than for the 1′-hydroxylation of the 2′,3′-oxides of safrole and estragole, although rat liver microsomes epoxidized 1′-hydroxysafrole as well or better than they hydroxylated safrole-2′,3′-oxide. With the exception of the 1′-hydroxylation of estragole by liver microsomes from male mice, the rates of 1′-hydroxylation and of 2′,3′-epoxidation of estragole derivatives were greater than for the safrole derivatives. In general, the rates of epoxidation of safrole, estragole, and their derivatives were greater for liver microsomes from female, rather than male, mice and from male, rather than female, rats.

The 2′,3′-epoxidation of eugenol was just detectable (<1 nmol/mg protein/h) under the conditions of these assays and was generally no more than 2% of the rates observed with estragole. *trans*-Anethole was hydroxylated at the 3′-position at relatively high rates (63—270 nmol/mg protein/h), which generally

TABLE I

YIELDS OF HYDROXY AND EPOXY ALKENYLBENZENE DERIVATIVES FORMED BY MOUSE AND RAT LIVER MICROSOMES FORTIFIED WITH AN NADPH-GENERATING SYSTEM

The incubation mixture is described in Materials and Methods. Mean ± standard deviation; numbers in parentheses are the numbers of pools of mouse liver (5—18 livers per pool) or numbers of individual rat livers analyzed. Each value has been corrected for the loss during a 20-min incubation of the product from an identical reaction mixture from which the substrate was omitted. The corrections were made on the basis of the demonstrated metabolite formation via zero-order kinetics and the loss of the products via first-order kinetics; the extraction of the metabolic products was nearly quantitative. The amounts of the 2′,3′-oxide of safrole, estragole, 1′-hydroxysafrole, and 1′-hydroxyestragole added to the flasks for determination of recovery and the percent recovered at the end of the incubations are given in Table III. For the other metabolites, the amounts added and the recoveries with female mouse liver were as follows: 1′-hydroxysafrole, 200 nmol, 81%; 1′-hydroxyestragole, 120 nmol, 84%; *trans*-3′-hydroxyanethole, 600 nmol, 91%; and eugenol-2′,3′-oxide, 60 nmol, 79%. The recoveries with male mouse and male and female rat liver microsomes were similar.

Substrate	Product	Yield (nmol product formed per mg protein per h)			
		Mouse		Rat	
		Male	Female	Male	Female
Estragole	1′-Hydroxyestragole	44 ± 10 (4)	138 ± 19 (6)	74 ± 10 (4)	42 ± 4 (3)
Estragole	Estragole-2′,3′-oxide	53 ± 3 (4)	70 ± 12 (6)	100 ± 10 (4)	27 ± 5 (3)
1′-Hydroxyestragole	1′-Hydroxyestragole-2′,3′-oxide	11 ± 2 (3)	18 ± 3 (5)	21 ± 3 (4)	5.3 ± 0.3 (3)
Estragole-2′,-3′-oxide	1′-Hydroxyestragole-2′,3′-oxide	44 ± 13 (4)	110 ± 28 (3)	68 ± 21 (4)	19 ± 8 (4)
Safrole	1′-Hydroxysafrole	85 ± 21 (3)	82 ± 18 (5)	23 ± 3 (4)	17 ± 2 (4)
Safrole	Safrole-2′,3′-oxide	10 ± 3 (3)	15 ± 2 (5)	14 ± 1 (4)	3.0 ± 0.7 (4)
1′-Hydroxysafrole	1′-Hydroxysafrole-2′,3′-oxide	2.2 ± 0.7 (4)	4.5 ± 1.0 (6)	10 ± 4 (3)	1.6 ± 0.4 (4)
Safrole-2′,3′-oxide	1′-Hydroxysafrole-2′,3′-oxide	7.6 ± 0.6 (3)	18 ± 6 (5)	3.3 ± 0.7 (4)	1.7 ± 0.4 (5)
Eugenol	Eugenol-2′,3′-oxide	0.7 ± 0.3 (7)	≤1.3 * (4)	≤1.9 * (4)	0.4 ± 0.1 (4)
trans-Anethole	*trans*-3′Hydroxyanethole	130 ± 27 (4)	95 ± 18 (4)	270 ± 23 (4)	63 ± 8 (6)

* Value was the limit of detection for the experiment

exceeded those observed for the 1′-hydroxylation of either estragole or safrole. The 1′,2′-epoxidation of anethole and the 1′-hydroxylation of eugenol were not investigated, because of the unavailability of standard compounds. Attempts to synthesize anethole-1′,2′-oxide were unsuccessful, possibly owing to the reactivity of the epoxide or of an intermediate in its synthesis.

Since the 2′,3′-oxides of safrole, estragole, and eugenol were very susceptible to hydrolysis by microsomal epoxide hydrase (EC 3.3.2.3) (Ref. 20; see below), trichloropropylene oxide was added to all of the reaction mixtures to inhibit the hydrase. Trichloropropylene oxide has been reported to inhibit rat liver microsomal cytochrome P-450-linked mixed function oxygenase activities toward some substrates [22], but no significant inhibition by trichloropropylene oxide at the levels used was observed toward side-chain hydroxylation of any of the substrates or toward epoxidation of 1′-hydroxysafrole or 1′-hydroxyestragole. Little or no safrole- or estragole-2′,3′-oxide was detected in the absence of trichloropropylene oxide, but 3—15 and 27—100 nmol/mg protein/h were obtained when trichloropropylene oxide was added to incubation mixtures containing safrole and estragole, respectively.

The formation of these hydroxy and epoxy metabolites appeared to occur via a microsomal system that was dependent on cytochrome P-450 and NADPH (Table II). Addition of 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine, a specific cytochrome P-450 inhibitor [23], to the incubation mixture reduced the levels of these metabolites to 2—12% of the values observed in the absence of the inhibitor. In the absence of an NADPH-generating system, the amounts of these products were negligible, and other studies showed that both the hydroxylation and epoxidation of estragole were negligible in the absence of air.

We were specifically interested in the side-chain hydroxy and epoxy derivatives, and therefore only these metabolites were identified and quantitated.

TABLE II

DEPENDENCE OF THE MICROSOMAL HYDROXYLATION AND EPOXIDATION OF THE ALKENYLBENZENES ON NADPH AND CYTOCHROME P-450

The control values for each reaction were in the range of values shown in Table I. Hepatic microsomes from female mice were used. DPEA, 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine hydrobromide, which was a gift from Dr. R.E. McMahon of the Eli Lilly Co., Indianapolis, Ind., was added at a final concentration of 1 mM. The cofactors omitted were NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. See the Materials and Methods. Each value in the final column is the limit of detection for the metabolite for the experiment.

Substrate	Product	% of control reactions	
		+ DPEA	—Cofactors
Estragole	1′-Hydroxyestragole	5	≤0.6
Estragole	Estragole-2′,3′-oxide	12	≤1
1′-Hydroxyestragole	1′-Hydroxyestragole-2′,3′-oxide	6	≤0.8
Estragole-2′,3′-oxide	1′-Hydroxyestragole-2′,3′-oxide	2	≤0.3
Safrole	1′-Hydroxysafrole	4	≤0.2
Safrole	Safrole-2′,3′-oxide	8	≤0.7
1′-Hydroxysafrole	1′-Hydroxysafrole-2′,3′-oxide	11	≤0.8
Safrole-2′,3′-oxide	1′-Hydroxysafrole-2′,3′-oxide	3	≤0.4
trans-Anethole	*trans*-3′-Hydroxyanethole	3	≤3

However, the patterns obtained on HPLC of the extracts of the incubation mixtures indicated that some other products were present. On the basis of the peak heights at the wavelengths at which the columns were monitored (254 nm for anethole derivatives and 280 nm for all other compounds), these unidentified products accounted for 20—60% of the products observed from safrole, estragole, and their hydroxy or epoxy derivatives. 3'-Hydroxyanethole was essentially the only product of anethole observed, while eugenol-2',3'-oxide, a trace metabolite, was accompanied by other products formed in larger amounts.

Susceptibilities of the 2',3'-oxides and 1'-hydroxy-2',3'-oxides to microsomal epoxide hydrase

In agreement with earlier studies on the high susceptibility of safrole-2',3'-oxide to epoxide hydrase in rabbit liver microsomes [20], safrole-2',3'-oxide and estragole-2',3'-oxide were very susceptible to hydrolysis by rat and mouse liver microsomal epoxide hydrases. Thus, after incubation for 20 min of 80—100 nmol of safrole-2',3'-oxide in the usual incubation system with either rat or mouse liver microsomes in the absence of trichloropropylene oxide, less than 5% of the epoxide was recovered (Table III). Similar results were obtained on incubation of estragole-2',3'-oxide. Addition of 4 mM trichloropropylene oxide permitted recovery of 50—80% of these oxides with either rat or mouse liver microsomes (Table III); similar data were obtained on addition of 8 mM trichloropropylene oxide. Analysis of the extracts of the incubation mixtures by HPLC (μBondapak C18 reverse-phase column eluted with acetonitrile/water) showed polar metabolites that co-chromatographed with synthetic safrole- or estragole-2',3'-dihydrodiol. In the absence of trichloropropylene oxide 70—90% of safrole-2',3'-oxide and 30—85% of estragole-2',3'-oxide were recovered as the dihydrodiols. On addition of 4 mM trichloropropylene oxide only 2—6% of each of the epoxides was recovered as the corresponding dihydrodiol.

Eugenol-2',3'-oxide was also very susceptible to hydrolysis by microsomal epoxide hydrase. For example, on addition of this oxide to female mouse liver microsomes only about 7% was recovered when no trichloropropylene oxide was included in the incubation mixture, while about 70% was recovered on addition of 4 mM trichloropropylene oxide.

In contrast, the recoveries of 1'-hydroxysafrole- and 1'-hydroxyestragole-2',3'-oxide after incubation for 20 min in the absence of trichloropropylene oxide were 50—80%. Addition of 4 mM trichloropropylene oxide generally resulted in recoveries of these two epoxides of at least 90%. HPLC of the extracts of the incubation mixtures from the 1'-hydroxy epoxides showed little or no formation of new polar compounds in the presence or absence of trichloropropylene oxide. However, the synthetic 1',2',3'-trihydrotriols were not available as standards.

Electrophilic reactivities of the 2',3'-oxides

Although they are relatively stable epoxides, the 2',3'-oxides of safrole, 1'-hydroxysafrole, estragole, 1'-hydroxyestragole, and eugenol reacted non-enzymatically with guanosine at neutral pH (Table IV). Under the conditions of

TABLE III

EFFECT OF TCPO ON THE RECOVERIES OF THE 2′,3′-OXIDES AND 1′-HYDROXY-2′,3′-OXIDES OF SAFROLE AND ESTRAGOLE INCUBATED WITH RAT OR MOUSE LIVER MICROSOMES

The incubation mixture and conditions are described in the Materials and Methods. 80—100 nmols of epoxide were added per flask. TCPO was added in ethanol at a final concentration of 4 mM; all flasks contained a final ethanol concentration of 3%. Each flask contained 2 mg of microsomal protein/ml of reaction mixture. Each value is the mean ± standard deviation, unless otherwise indicated. TCPO, trichloropropylene oxide. Numbers in parentheses indicate numbers of individual rat livers or pools of 5-17 mouse livers. The same livers were always analyzed both with and without TCPO.

Species	Sex	Percent recovered after incubation for 20 min							
		Estragole-2′,3′-oxide		1′-Hydroxyestragole-2′,3′-oxide		Safrole-2′,3′-oxide		1′-Hydroxysafrole-2′,3′-oxide	
		+TCPO	—TCPO	+TCPO	—TCPO	+TCPO	—TCPO	+TCPO	—TCPO
Mouse	F	50 ± 4	≤5 (3) *	90 ± 6	63 ± 18 (7)	71 ± 5	≤5 (3) *	92 ± 9	77 ± 16 (6)
	M	44 ± 14	≤1 (3) *	100 ± 17	71 ± 7 (4)	57 ± 15	≤1 (3) *	99 ± 4	69 ± 10 (3)
Rat	F	83; 82 **	≤1 (2) *	80 ± 13	68 ± 3 (3)	73; 80	≤1 (2) *	100 ± 2	83 ± 12 (3)
	M	75; 54	≤1 (2) *	100; 106	42; 73 (2)	55; 48	≤1 (2) *	100 ± 6	53 ± 3 (3)

* Value was the limit of detection for the experiment.

** Individual values.

TABLE IV

REACTIVITIES OF 2',3'-OXIDES WITH GUANOSINE

See Materials and Methods for procedure. Each value given is the mean ± standard deviation for 4 experiments.

Compound	Percent of guanosine reacted	
	25 h	97 h
Safrole-2',3'-oxide	6 ± 1	20 ± 2
1'-Hydroxysafrole-2',3'-oxide	5 ± 2	23 ± 2
Estragole-2',3'-oxide	6 ± 0.5	20 ± 0.4
1'-Hydroxyestragole-2',3'-oxide	9 ± 1	27 ± 0.6
Eugenol-2',3'-oxide	5 ± 0.5	17 ± 2

the assay (a 50-fold molar excess of an oxide and 50% acetone to facilitate its solution), 5—9% of the guanosine was converted to products within 24 h, and the reactions proceeded approximately linearly for 97 h. Similar results have been reported previously for safrole- and 1'-hydroxysafrole-2',3'-oxide [11].

Analysis of the reaction mixtures by HPLC at 97 h showed the presence of one major and two minor products from 1'-hydroxyestragole-2',3'-oxide and 1'-hydroxysafrole-2',3'-oxide. Resolution of the reaction mixtures from safrole-, estragole-, and eugenol-2',3'-oxide gave evidence for one major product and one or two minor products in each case. The breadth and/or asymmetry of some of the peaks suggested that they might contain more than one product.

Discussion

Quantitation of the rates of formation of the 1'-hydroxy derivatives, 2',3'-oxides, and 1'-hydroxy-2',3'-oxides of safrole and estragole by rat and mouse liver microsomes extends earlier data on the formation of the 1'-hydroxy derivatives in vivo by rats and mice [6,9] and on the detection of the 2',3'-dihydrodiol and 1',2',3'-trihydrotriol of safrole in the urine of rodents treated with safrole and in safrole-treated liver cultures [12,13]. Since 1'-hydroxysafrole and 1'-hydroxyestragole are more potent carcinogens than the parent compounds [5,6,10], their formation in relatively large amounts both in vivo and in vitro indicates that they are important intermediates in the metabolic activation of safrole and estragole. The relatively high rates of formation of the 2',3'-oxides, which are electrophilic and relatively strong direct mutagens [10,14,15], suggest that they could be important to the carcinogenic processes induced by the parent compounds. However, their high susceptibility to hepatic microsomal epoxide hydrase may limit the amounts that are available for reaction with macromolecules within the hepatic cells. Judging from the rates of formation in vitro, the 1'-hydroxy-2',3'-oxides may be formed in smaller amounts in vivo than the parent 2',3'-oxides, but the relative insusceptibility of the 1'-hydroxy epoxides to microsomal epoxide hydrase would appear to favor their persistence in cells. Studies on the susceptibilities of these 2',3'-oxides to the cytoplasmic epoxide hydrase [24] are needed for further assessment of this problem.

Although several-fold differences were found between male and female or rat and mouse liver microsomes in their activities for some of these reactions, the data are difficult to interpret in terms of the available tumor incidence data. When fed as 0.5% of the diet, 1′-hydroxysafrole induced hepatic tumors more readily in male rats than in female mice [5,10]. Male mice did not develop hepatic tumors under these conditions, but the median survival time was less than for the female mice.

The marked difference in susceptibility of the 2′,3′-oxides and the 1′-hydroxy-2′,3′-oxides of safrole and estragole to hydrolysis by hepatic microsomal epoxide hydrase is analogous to the much greater resistance of (±)-7β,8α-dihydroxy-9β,10β-epoxy- and (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene as compared to that of 9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [25]. These data suggest that a hydroxyl group vicinal to the epoxide ring confers resistance of the epoxide to hydrolysis by microsomal epoxide hydrase. This marked difference in epoxide hydrase susceptibility of the 2′,3′-oxides of safrole and estragole may be relevant to the carcinogenicities of the 2′,3′-oxides. Thus, although the 2′,3′-oxides of 1′-hydroxysafrole and 1′-hydroxyestragole are less active as direct-acting mutagens than are the 2′,3′-oxides of safrole and estragole [15], the 1′-hydroxy-2′,3′-oxides are as active or more active as initiators of papilloma formation in mouse skin than are the 2′,3′-oxides (Ref. 10 and Swanson, Miller, and Miller, unpublished data).

In addition to the epoxides, safrole and estragole are also metabolized to other electrophilic reactants. Evidence has been presented for the urinary excretion by rats treated with safrole or estragole of small amounts of tertiary aminopropiophenones (Mannich bases) presumably derived from 1′-oxosafrole and 1′-oxoestragole [26,27]. Liver cytosols from rats and mice converted 1′-hydroxysafrole to an electrophilic reactant (presumably the 1′-sulfuric acid ester) in a 3′-phosphoadenosine-5′-phosphosulfate-dependent reaction [11]. Furthermore, recent data from this laboratory suggest that esters of 1′-hydroxyestragole and 1′-hydroxysafrole are the major electrophilic reactants in the mouse liver in vivo, since the hepatic DNA adducts formed from 1′-hydroxyestragole and 1′-hydroxysafrole showed solvent partition as a function of pH and chromatographic properties identical to those of adducts formed from the model electrophiles 1′-acetoxyestragole or 1′-acetoxysafrole and DNA in vitro (Ref. 28 and Phillips, Miller, Miller, and Adams, unpublished data).

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