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Investigation of the role of the 2′,3′-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs

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Abstract

The genotoxic potential of naturally occurring allylbenzene analogs, including safrole, eugenol, estragole, and others, has been examined in many studies over the past 30 years. It has been established that these compounds are subject to biotransformation in the liver, which can lead to the formation of reactive electrophilic intermediates. The major route of bioactivation is via hydroxylation of the 1′ carbon atom of the allylic side chain. We have synthesized 2′,3′- (allylic) epoxide derivatives of allylbenzene, estragole eugenol and safrole, and have used them to characterize the genotoxic potential of epoxidation at the allylic double bond for allylbenzene and its naturally occurring analogs. In order to assert that this pathway has the potential for genotoxicity, it is necessary to demonstrate (1) that epoxide metabolites of these compounds are capable of forming covalent adducts with DNA bases; and (2) that these epoxide metabolites are actually formed in vivo. We have demonstrated that allylic epoxides derived from allylbenzene and estragole are capable of forming covalent adducts with all four deoxyribonucleotides in vitro and, in the case of deoxyguanosine, form at least four different adducts. We also deduce, from evidence obtained using the isolated perfused rat liver, that formation of potentially genotoxic 2′,3′ epoxide metabolites occurs readily in vivo, but that these metabolites are rapidly further metabolized to less toxic dihydrodiol or glutathione conjugates. We conclude that 2′,3′ epoxide metabolites of allylbenzene analogs are formed in vivo and that these epoxides are sufficiently reactive to facilely form covalent bonds with DNA bases. Epoxide formation at the allylic double bond represents, therefore, a potentially genotoxic bioactivation pathway for allylbenzene analogs. However, comparison of the relative kinetics of epoxide metabolism and epoxide formation suggests that a wide margin of protection from DNA covalent adduct formation exists in the rat liver, thus preventing genotoxicity resulting from this pathway to any significant degree. In this regard, we have also observed that the general rate of epoxide hydrolysis is much greater in human liver than in rat liver. We therefore suggest that while the epoxidation pathway poses a potential genotoxic threat to humans, no actual genotoxicity occurs as a result of this metabolic pathway.

Keywords: Bioactivation; Dietary allylbenzene analogs; 2′,3′-Epoxidation pathway; Genotoxicity

Abbreviations: ABO, allylbenzene 2′,3′-oxide; ESO, estragole 2′,3′-oxide; GC/MS, gas chromatograph-linked mass spectroscopy; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography..

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1. Introduction

Safrole, estragole and eugenol are natural product analogs of allylbenzene (Fig. 1) that are present in many dietary plants and their extracts. These compounds are present in particularly high concentrations in numerous herbs and spices, such as basil, cinnamon, nutmeg, ginger, black pepper, clove, tarragon, and many others (Swanson et al., 1979; Miller et al., 1983; Woo et al., 1988). The genotoxicity of these compounds has been well studied and documented for over 40 years (Borchert et al., 1973a,b; Drinkwater et al., 1976; Ioannides et al., 1981; Woo et al., 1988; Howes et al., 1990; Chan and Caldwell, 1992). The accepted mechanism for the genotoxicity of these compounds involves cytochrome P450-catalyzed hydroxylation at the 1 carbon atom, followed by the formation of a labile ester, primarily through sulfation. This ester spontaneously decomposes to produce a highly electrophilic carbonium cation, which can form covalent adducts with cellular macromolecules (Fig. 2) (Borchert et al., 1973a,b; Wislocki et al., 1977; Swanson et al., 1979; Ioannides et al., 1981; Chan and Caldwell, 1992).

In the process of elucidating the mechanism of genotoxicity of these compounds, other potentially toxic intermediates in addition to the 1 hydroxy metabolite have been identified. In particular, the potential of the allylbenzene analogs

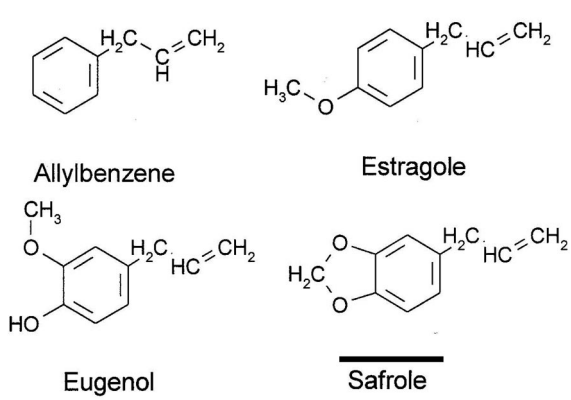

Fig. 1. Structures of allylbenzene and its naturally occurring analogs estragole, eugenol and safrole.

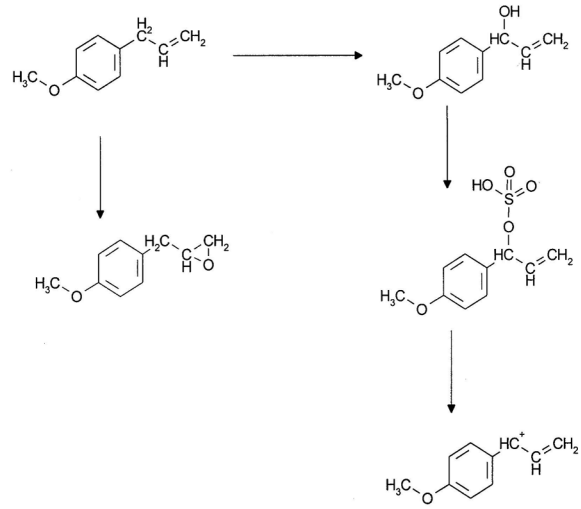

Fig. 2. Metabolic activation of an allylbenzene analog (estragole). Two potential bioactivation pathways are shown: on the left, initiated by epoxidation at the 2′,3′ double bond; on the right, initiated by hydroxylation at the 1′ carbon atom.

for forming stable epoxides at the 2′,3′ double bond has attracted attention. The structural features of these compounds predict that oxygenation of this isolated double bond should produce an epoxide that is sufficiently reactive to form covalent adducts with macromolecular nucleophiles, yet sufficiently stable to migrate away from its site of formation and produce damage at distant cellular or nuclear sites. In fact, epoxide formation has been inferred (Solheim and Scheline, 1973; Stillwell et al., 1974; Delaforge et al., 1978, 1980) from the detection of dihydrodiols, which are formed by further metabolism of epoxides, but the epoxide metabolites themselves have not been isolated in significant quantities (Solheim and Scheline, 1973; Delaforge et al., 1980; Swanson et al., 1981). However, no DNA adducts have been identified for which an epoxide metabolite precursor can be rationalized. No evidence has yet been produced implicating the epoxidation pathway as a contributor to genotoxicity for this family of compounds, even though their structural similarity to compounds such as styrene would make epoxidation a very logical pathway for genotoxic bioactivation.

The apparent absence of a role for the epoxidation pathway in the genotoxicity of this family of compounds might be explained by any of three hypotheses. These hypotheses propose that: (1) Epoxidation may not occur to any significant degree in vivo, and the reason that no evidence has been obtained for genotoxicity of epoxide intermediates is that they are not formed to any significant degree in the first place; (2) epoxides may be formed in vivo, but they may be extremely chemically stable and insufficiently electrophilic to form covalent adducts with cellular macromolecules; or (3) epoxide metabolites derived from these compounds may be formed in vivo and are sufficiently electrophilic to form covalent adducts with proteins and DNA, but the detoxification of these epoxides is sufficiently rapid and efficient, so that no significant net accumulation of the reactive epoxide occurs.

In studies investigating the existence of multiple epoxide hydrolases within the cell, Hammock and coworkers (Ota and Hammock, 1980) demonstrated that allylbenzene 2′,3′-oxide (ABO) is an excellent substrate for both the microsomal and the soluble epoxide hydrolases. These results suggested that the allylic epoxides might be very efficiently detoxified in the cell, lending initial credence to the third hypothesis. We therefore undertook a more thorough investigation of capability of the cell to detoxify these allylic epoxides and attempted to extend these studies to provide a more definitive assessment of the role of the formation of allylic epoxides in the overall genotoxicity of allylbenzene analogs. In so doing, we feel that we have demonstrated an example of a highly reactive genotoxic intermediate that is readily formed in vivo, but is, at the same time, subject to very rapid and efficient detoxification. This efficient detoxification creates a large safety 'threshhold', such that production of reactive epoxide intermediates under any observed physiological condition produces no net genotoxicity. We feel that this is an excellent example of the lack of applicability of the 'zero threshhold dose' principle to every compound or intermediate that is identified as potentially genotoxic by in-vitro analysis.

2. Materials and methods

Commonly available organic and biochemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis MO). Precursors for radiochemical synthesis were obtained from ICN Biochemicals (Irvine, CA). Animal tissue for fractionation was obtained frozen from Pel-Freez Biologicals (Rogers, AK). Adult male Sprague–Dawley rats were obtained from Sasco/King Laboratories (Madison, WI). Human liver samples were obtained as surgical excess during an exploratory biopsy for metastatic cancer at the University of Illinois Hospital. All tissue was frozen in liquid nitrogen, and only those samples proven to be free of cancer were used in this study. Homogeneous microsomal epoxide hydrolase was prepared from rat liver, as previously described (Lu et al., 1975). The synthesis of radiolabeled epoxide derivatives of allylbenzene, estragole, eugenol, and safrole has also been described (Luo et al., 1992).

The reaction in vitro between epoxides and deoxyribonucleotides and nucleosides has been described (Luo and Guenthner, 1996). Thin-layer chromatographic separation and subsequent analysis of adducts was carried out on 200 μm silica gel-coated plates, developed with 80/10/25 butanol/ethyl ether/water. Covalent adducts of epoxides with salmon sperm DNA were produced as described previously (Luo and Guenthner, 1996). They were isolated and separated by reverse-phase HPLC, using methanol/water gradients. Products for spectroscopic analysis by NMR or GC/MS were purified by preparative silica gel TLC plates developed in 80/20 hexane/ether. Mass spectroscopy was performed using a Finnigan model 4510 mass spectrometer operating in negative ion fast bombardment mode. The sample was introduced by direct insertion. One-dimensional proton and ^{13}C NMR spectra were determined using a Nicolet NMC 360 Spectrometer as described previously (Luo and Guenthner, 1996).

The preparation of human and animal tissue samples, and the determination of their epoxide hydrolase activities, utilizing radiolabeled epoxide substrates, were performed by previously described methods (Luo et al., 1992). The techniques

involved in perfusion of isolated rat livers with epoxides or parent compounds, and the subsequent separation and analysis of metabolites in the perfusate, have been thoroughly described in an earlier publication (Luo and Guenthner, 1995).

3. Results

Radiolabeled ABO and its analogs were synthesized as described previously (Luo et al., 1992). The specific radioactivity of these compounds was sufficiently high to allow recovery and quantitation of radiolabeled epoxide-nucleotide adducts following incubation of the epoxides with calf thymus DNA in vitro. Fig. 3A–C shows HPLC radiochromatograms of covalent nucleotide adducts with ABO, formed either in the presence (Fig. 3B) or absence (Fig. 3A) of purified rat microsomal epoxide hydrolase in the incubation mixture. At least two covalent adduct peaks are seen, both of which are eliminated in the presence of microsomal epoxide hydrolase. Similar results were seen with estragole 2′,3′-oxide (ESO). The rate of adduct formation with ABO was estimated to be 100 pmol/mg/h. This is a similar rate to that seen for the formation of covalent adducts of DNA in vitro with a similar aflatoxin B1 epoxide (Essigmann et al., 1977) or styrene 1′,2′ oxide (Hemminki and Hesso, 1984; Cantoreggi and Lutz, 1992) under similar conditions. These results indicate that DNA adducts are readily formed with these allylic epoxides. Furthermore, the ability of added homogeneous microsomal epoxide hydrolase to prevent this adduct formation (Fig. 3B) suggests that the epoxide function is a probable site of adduct formation and that these epoxides are good substrates for microsomal epoxide hydrolase. Fig. 3C shows adduct formation in the presence of rat-liver cytosol. Again, adduct formation is prevented, most likely by a combination of soluble epoxide hydrolase and GSH transferase activity.

Confirmation of the ability of micro somal and soluble epoxide hydrolases to catalyze the detoxification of allylic epoxides, by converting them to dihydrodiols, is provided in Table 1. The K_m for these compounds is in the millimolar range, and the V_{max}, expressed as moles product formed per mg cell fraction protein per min, is in the micromolar range. In both respects, the activities of the epoxide hydrolases toward these allylic epoxides are similar to the hydrolase activity toward styrene oxide (Luo et al., 1992).

Large-scale reactions of non-radiolabeled allylic epoxides with deoxyribonucleosides were carried out in vitro. The time course of the formation of the four ABO-dG adducts was followed. The adducts were designated as I–IV according to their increasing Rf values in the TLC system. Fig. 4 shows that adduct 1 is rapidly formed but unstable, suggesting that it is probably an N7 adduct of dG (Tomasz, 1970; Margison and Pegg, 1981). Adducts II, III and IV are formed relatively more slowly, and their levels increased throughout the period of analysis. This suggests that they may be O6, N1 or N2 adducts. Adduct III was selected for further characterization, primarily because it was formed in the greatest abundance at 72 h, and also because it was most easily recovered from the TLC plate without contamination by other adducts.

Fig. 5 shows the results of negative ion FAB mass spectroscopy of adduct III. The spectrogram is consistent with a single adduct of ABO and dG. Although this provides proof that the isolated compound is, in fact, a covalent adduct, the actual structure of the adduct, and particularly the molecular sites of adduct formation, cannot be deduced from this evidence alone. Therefore, adduct III was subjected to ^{13}C and ^{1}H NMR spectroscopy, in order to obtain a definitive structural analysis. Table 2 is a summary of the important features of ^{1}H NMR spectroscopic analysis of adduct III and related compounds. Assignment of the peaks has been previously described (Luo and Guenthner, 1996). This analysis provides unequivocal evidence that adduct III is formed at the N1 nitrogen of dG, as drawn. The primary features of the adduct III NMR spectrum that lead to this conclusion are: disappearance of the N1 proton, and a 0.6 ppm shift in the N2 proton, both of which predict an N1, but not an N2, adduct, and the absence of a shift in the C8 proton, which eliminates the possibility of an N7 adduct. Tautomerism of an O6 adduct would also

lead to the loss of the N1 proton, but not the observed shift of the N2 proton. Thus, the assignment of the structure adduct III as an N1 adduct is fully supported by the ^{1}H NMR analysis, while the evidence eliminates the possibilities that adduct III is an N2, N7 or O6 adduct. The conclusion that the adduct is formed with the 3′-carbon of ABO is supported by the ^{13}C NMR spectrum (Luo and Guenthner, 1996).

Several pieces of evidence suggest, therefore, that ABO and its natural product-derived epoxide analogs are chemically reactive and capable of

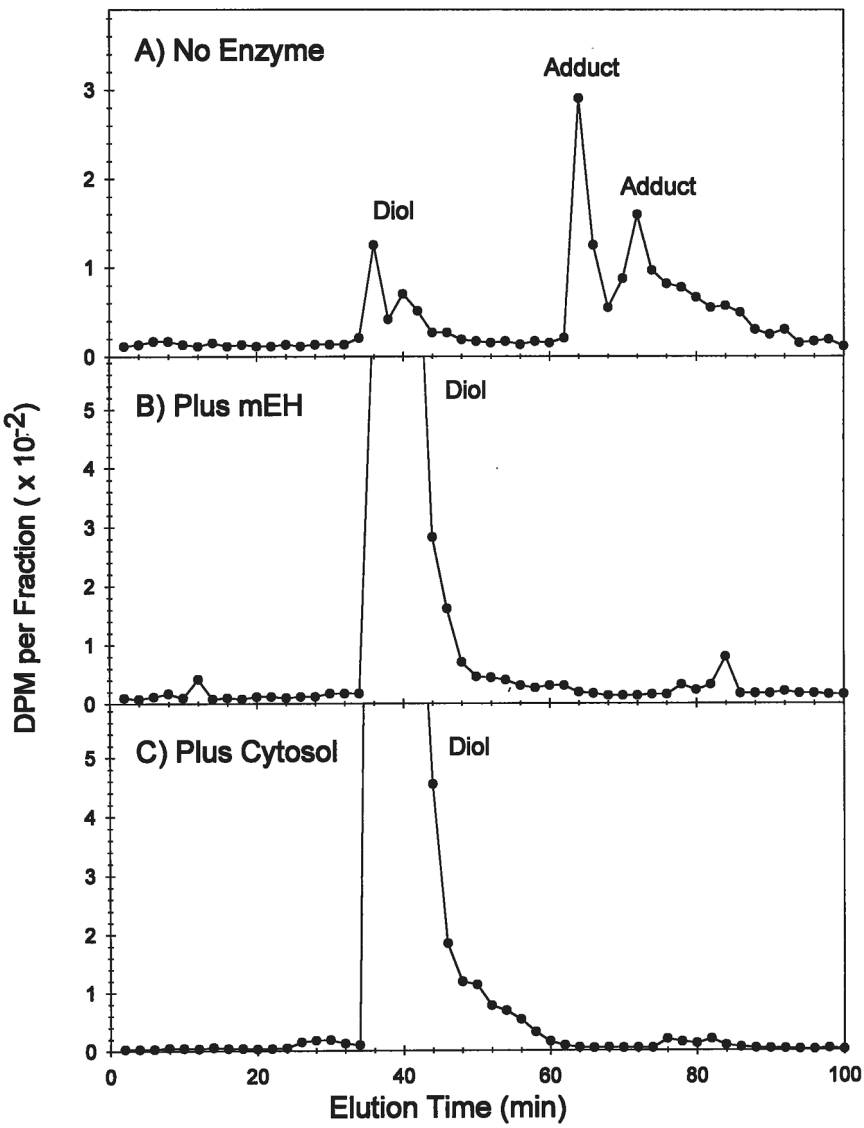

Fig. 3. HPLC analysis of ABO-nucleotide adducts formed in the presence and absence of added detoxifying enzymes. Radiolabeled ABO was reacted with salmon sperm DNA for 48 h at 37%. To the incubations shown in (B) and (C), purified microsomal epoxide hydrolase or unfractionated mouse-liver cytosol protein was added. The DNA was hydrolyzed, and nucleotides were separated by reverse-phase HPLC. Radiolabeled products were quantitated by scintillation counting of collected fractions. Peaks were identified by the corresponding elution times of synthetic standards.

Table 1
Microsomal and soluble epoxide hydrolase activity towards allylbenzene 2′,3′-oxide and its naturally occurring analogs[a]

	Microsomal EH	Soluble EH
Allylbenzene oxide	$K_m = 2.93$	$K_m = 5.66$
	$V_{max} = 0.423$	$V_{max} = 0.553$
Estragole oxide	$K_m = 2.24$	$K_m = 0.920$
	$V_{max} = 0.833$	$V_{max} = 0.563$
Eugenol oxide	$K_m = 22.7$	$K_m = 1.77$
	$V_{max} = 1.97$	$V_{max} = 0.846$
Safrole oxide	$K_m = 0.197$	$K_m = 2.02$
	$V_{max} = 0.092$	$V_{max} = 0.310$

[a] K_m values are millimolar. V_{max} values are micromoles per minute per milligram of cellular protein. Values were determined using five or six substrate concentrations, and the mean activity of at least four replicate incubations was determined for each substrate concentration. Kinetic parameters were determined by fitting the hyperbolic curve, using a commercial statistics software program.

forming covalent adducts with DNA. The evidence also indicates that the formation of these adducts is efficiently prevented in vitro by microsomal and soluble epoxide hydrolases, and possibly by other cytosolic enzymes. These chemical studies suggest, therefore, that epoxide metabolites of allylbenzene and its related natural

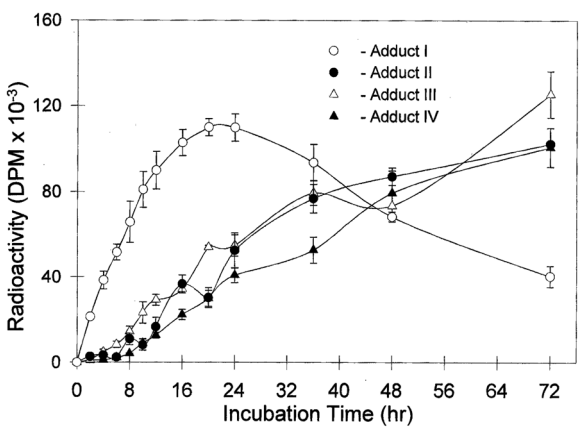

Fig. 4. Time course of adduct formation between ABO and deoxyguanosine. Adducts I–IV correspond to the relative Rf values in lane 9 of Fig. 4. ABO was incubated with [^{14}C-8] 2′-deoxyguanosine for up to 72 h. Aliquots were withdrawn at the indicated time points and analyzed by TLC. Amounts of individual adducts were quantitated by scraping them from the plates followed by scintillation counting.

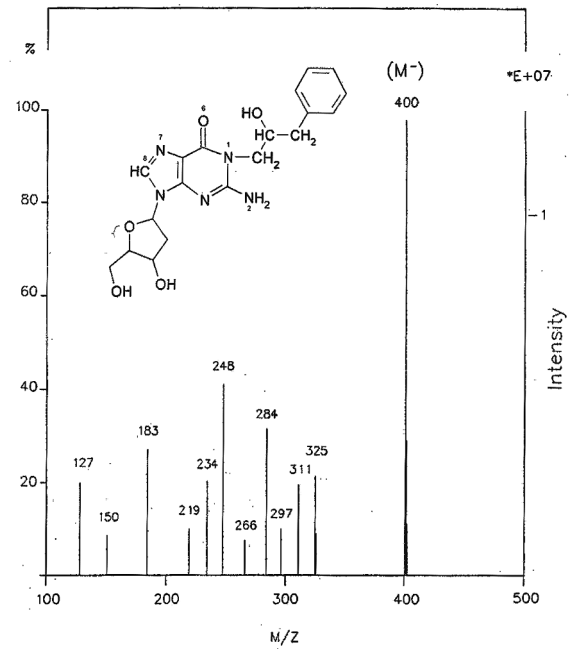

Fig. 5. Mass spectral analysis of ABO-dG adduct III. The spectrum obtained after negative ion fast atom bombardment is shown. Major peaks with mass >100 amu are shown. M^- indicates the calculated molecular mass ion -1 amu. The insert shows the putative structure of this adduct, consistent with this fragmentation pattern.

product analogs are highly reactive intermediates with a high potential for cytotoxicity and genotoxicity, and that the metabolic pathway for these that is initiated by epoxidation has an equivalent potential for biochemical damage to that posed by the 1-hydroxylation pathway. In order to assess the actual, in contrast to the potential, threat posed by the epoxidation pathway, we used the isolated perfused rat liver as a model for studying the toxicity of these epoxide intermediates in an intact biological system. The first set of these studies examined the clearance and metabolic fate of allylbenzene oxide and estragole oxide in the perfused rat liver. Upon perfusion of livers with epoxides, we were able to recover two additional products in the perfusate. The first peak corresponded to dihydrodiol standards that were chemically synthesized. The second major peak was subjected to positive and negative ion mass spectroscopy and shown to have the struc-

ture of a glutathione conjugate at the C2 allylic carbon. Using HPLC to separate and quantitate these products, we were able to examine the relative rates of their formation by the isolated perfused rat liver, and thereby measure the metabolic clearance of the epoxides.

Fig. 6 shows the clearance of epoxides by the isolated perfused rat liver, and the formation of dihydrodiol and GSH conjugate metabolites. When low concentrations, 0.8 mM, of ABO or ESO (not shown) were added to the perfusate, the epoxides were almost immediately taken up by the livers and converted to both metabolites, with hydrolysis (dihydrodiol formation) predominating. The concentrations used approximate the K_m of the epoxide hydrolase and thus are sub-saturating. At higher concentrations of both epoxides, 8 mM, the K_m of the epoxide hydrolase is exceeded approximately five times. A biphasic elimination curve is seen, obeying zero-order kinetics at higher epoxide concentrations during the first 20 min and first-order kinetics as the concentration of epoxide falls. Again, both metabolites are formed, with the dihydrodiol predominating. This figure shows that even at very high concentrations of epoxides, detoxification proceeds rapidly and efficiently.

Fig. 7 shows the results obtained when the parent compound allylbenzene was added to the perfusate. No epoxide formation was detected, but dihydrodiol formation was detectable and could be quantitated. In a similar fashion, perfusion with estragole resulted in the recovery of dihydrodiol metabolites, but no epoxide metabolites (data not shown). These results indicate that the epoxide is formed by the rat liver in vivo, since it is inferred to be the precursor of the detected dihydrodiol. However, the fact that no intact epoxide metabolite can be isolated or detected indicates that it is detoxified as rapidly as it is formed, probably primarily by hydrolysis to the dihydrodiol.

The cytotoxicities of allylbenzene, estragole, and their epoxide metabolites were measured using the same rat-liver perfusion model. Cytotoxicity was determined by measuring the release of alanyl aminotransferase activity into the perfusate following addition of the test compounds. No significant cytotoxicity by either allylbenzene 2′,3′-oxide or estragole 2′,3′-oxide was seen, even at the highest concentration perfused. Alanyl aminotransferase activity in the perfusate was not increased above control values. Detoxification appears to be very efficient for both epoxides. However, the parent compounds were cytosolic, at concentrations equivalent to those expected to generate low concentrations of epoxide. These results suggest that both allylbenzene and estragole are converted to cytosolic metabolites in vivo, but that these metabolites are not 2′,3′ epoxides. Table 3 shows the ability of perfused radiolabeled epoxides to form covalent adducts with liver protein and nucleic acids. Small amounts of adducts are detectable when total protein and RNA

Table 2
Proton NMR analysis of ABO-dG adduct III[a]

	dG	dG-ABO adduct	N1-Me-G	ABDIOL
N1	10.6	–	–	–
C8	7.83	7.89	7.75	–
N2	6.38	6.88	6.84	–
Aromatic	7.20, 7.14	–	–	7.24
Cl′	2.70	–	–	2.71
C2′	3.90	–	–	3.88
C3′	3.60	–	–	3.50

[a] Table entries indicate shifts in ppm from tetramethylsilane. Tested compounds and reference standards are indicated across the top row. They are: 2′-deoxyguanosine, Adduct III, *N*′-methyldeoxyguanosine, and allylbenzene 2′,3′-dihydrodiol. Entries in the left-hand column designate peaks assigned to the indicated dG carbon or nitrogen atoms (top 3), or allylbenzene carbon atoms (bottom 4).

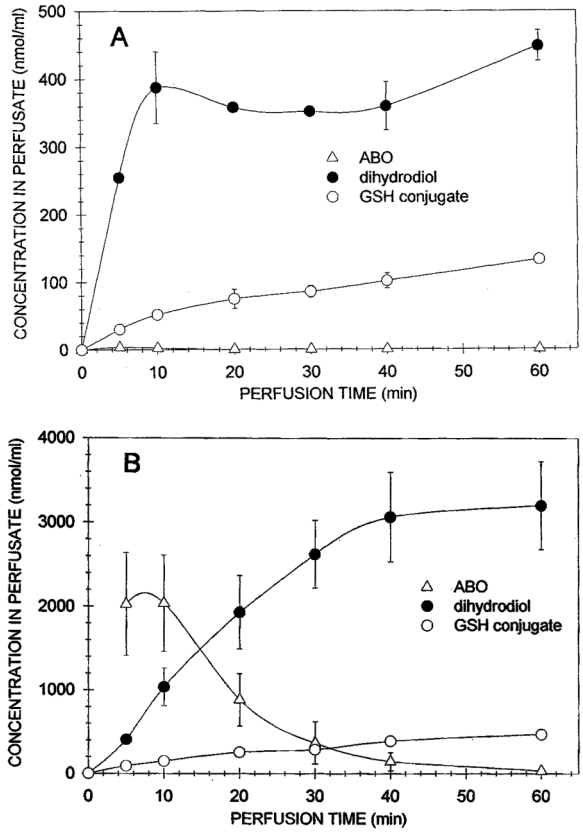

Fig. 6. Clearance of epoxides by isolated perfused rat liver and formation of subsequent metabolites. Isolated perfused rat livers were exposed to ABO at concentrations of 0.1 mmol/130 ml of perfusate (A) or 1.0 mmol/130 ml of perfusate (B). Aliquots of perfusate were withdrawn at the indicated time intervals, and the levels of parent epoxides and metabolites were measured by analytical HPLC. Compounds were identified by the corresponding elution times of synthetic standards. Points represent mean ± S.E. for three livers.

are isolated, but no DNA adducts can be detected in this perfusion system. These results again illustrate the efficiency of detoxification for potentially covalently binding epoxide metabolites, even when they are perfused at very high concentrations.

Finally, in order to estimate the relevance of the results obtained, both in vitro and with the perfused rat liver, to the risks posed by epoxide metabolites of allylbenzene analogs to humans, we compared the abilities of liver homogenates obtained from several species to detoxify allylbenzene and estragole oxides. As Fig. 8 shows, human livers have by far the highest allylic epoxide hydrolase activity of the species tested. However, human GSH transferase activities toward these substrates are relatively modest. Because human

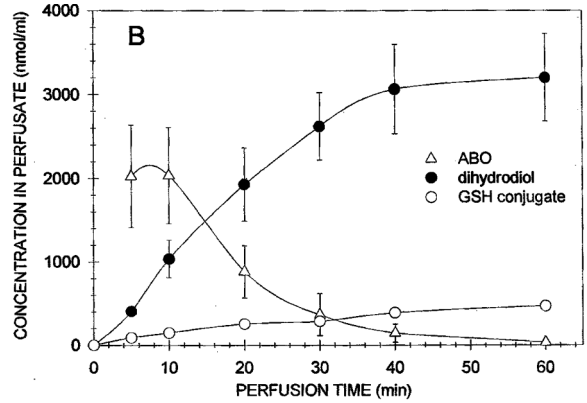

Fig. 7. Clearance of allylbenzene by isolated perfused rat liver and formation of subsequent metabolites. Isolated perfused rat livers were exposed to allylbenzene at concentrations of 2 mmol/130 ml of perfusate. Aliquots of perfusate were withdrawn at the indicated time intervals, and the levels of the parent compounds and metabolites were measured by analytical HPLC. Compounds were identified by the corresponding elution times of synthetic standards. Points represent mean ± S.E. for three livers.

Table 3
Covalent binding of radiolabeled ABO or ESO to cellular macromolecules in isolated perfused rat livers[a]

Perfused compound (dose/130 ml of perfusate)	Binding (pmol/mg)		
	Protein	RNA	DNA
ABO (0.1 mmol)	33.4	8.9	ND
ABO (1 mmol)	402	42.3	ND
ESO (0.1 mmol)	32.4	ND	ND
ESO (1 mmol)	405	34.4	ND

[a] Livers were perfused for 60 min with the indicated concentrations of tritiated ABO or ESO. Livers were recovered and homogenized, and protein, DNA, and RNA were extracted by published methods (Irving and Veazey, 1968; VanAnda et al., 1979). Extracted macromolecules were quantitated by absorbance spectrometry and solubilized, and covalent binding of the radiolabeled epoxides was determined by liquid scintillation counting. Values are the means of three determinations. ND indicates no detectable radioactivity above counting background.

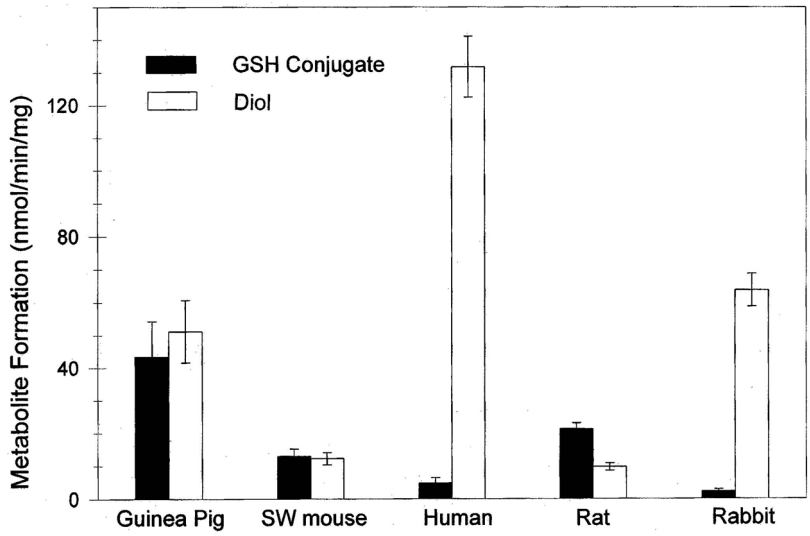

Fig. 8. Epoxide metabolizing activities of livers from obtained different species. Microsomes or cytosol were obtained from frozen livers derived from the indicated species. Epoxide hydrolase and glutathione 5-transferase activities were measured in vitro using radiolabeled ABO as substrate. Activities are means ± S.E. of three separate liver samples.

epoxide hydrolase activity is seven to 10 times higher than that seen in rat liver, we conclude that the level of protection against the cytotoxicity and genotoxicity of these epoxides in human liver is even greater than that seen in the isolated perfused rat liver. A very broad spread of epoxide hydrolase activities is seen in the human population. A 20-fold spread has been observed in the distribution of styrene oxide hydrolase activities from 20 human liver biopsy samples (Guenthner et al., 1993). Because the kinetics of allylic epoxide hydrolysis are similar to those for styrene oxide (Luo et al., 1992), we assume that these activities reflect relative ABO and ESO hydrolase activities as well. The hydrolase activity shown in Fig. 8 falls approximately in the middle of this distribution pattern. Furthermore, even though there is at least a 20-fold difference between individuals with the highest and lowest activities, humans with the lowest activities demonstrate epoxide hydrolase activity at least equivalent to that seen in rat liver, which offers full protection against epoxide cytotoxicity and genotoxicity.

4. Discussion

Previous studies have shown that natural product analogs of allylbenzene, such as safrole, estragole and eugenol, can be metabolically activated to toxic and genotoxic species (Borchert et al., 1973a,b; Drinkwater et al., 1976; Ioannides et al., 1981; Woo et al., 1988; Howes et al., 1990; Chan and Caldwell, 1992). It has been well established that the primary pathway of metabolic activation for these compounds is initiated by cytochrome P450-dependent hydroxylation at the 1′ carbon (Borchert et al., 1973a,b; Wislocki et al., 1977; Swanson et al., 1979; Ioannides et al., 1981; Chan and Caldwell, 1992). However, we and others have also shown that these compounds can be readily oxygenated at the 2′,3′ allylic double bond to form stable epoxides. These epoxide metabolites should, given their theoretical electrophilicity, also contribute to the overall genotoxicity of these compounds. However, no evidence has ever been provided to show that epoxidation represents a significant bioactivation pathway for these compounds in vivo. In light of the fact that the epoxidation pathway has not been implicated in the

genotoxicity of these compounds, in spite of the fact that epoxidation appears to occur readily in vitro, we developed three alternative hypotheses to explain this seeming contradiction. These hypotheses are that: (1) epoxidation does not occur to any significant degree in vivo; (2) epoxides may be formed in vivo, but they are stable and insufficiently electrophilic to form covalent adducts with cellular macromolecules; or (3) epoxide metabolites are formed in vivo, and are sufficiently electrophilic to form covalent adducts with proteins and DNA, but the detoxification of these epoxides is rapid and efficient, so that no significant accumulation of reactive epoxides occurs. We have provided evidence that the first two hypotheses are false, and that the third hypothesis explains the observed lack of epoxide-derived genotoxicity in vivo.

Epoxide metabolites of allylbenzene analogs are sufficiently electrophilic to readily form covalent adducts with DNA bases in vitro. As Figs. 3 and 4 show, allylbenzene 2′,3′ oxide and estragole 2′,3′ oxide form multiple covalent adducts (at least four in the case of allylbenzene oxide and deoxyguanosine) with DNA bases. This evidence demonstrates clearly that hypothesis (2), above, must be false. The proof that epoxide metabolites are formed in vivo, in contradiction to hypothesis (1), is somewhat less direct, but also quite clear. Earlier studies have provided strong indirect evidence that epoxide metabolites of allylbenzene analogs are formed in vivo. In feeding studies (Stillwell et al., 1974; Delaforge et al., 1980) or studies with isolated hepatocytes (Delaforge et al., 1978), only trace amounts of epoxide metabolites were found subsequent to exposure to safrole or estragole. However, dihydrodiol metabolites were recovered at significant levels in the urine of animals fed safrole or estragole (Solheim and Scheline, 1973; Stillwell et al., 1974). These dihydrodiol metabolites presumably represent end products of the epoxidation pathway, and accounted for up to 30% of the total metabolic clearance of estragole in one experiment (Solheim and Scheline, 1973). This is approximately the same contribution to the overall metabolic clearance of estragole provided by 1′ hydroxylation (Drinkwater et al., 1976). In our studies, dihydrodiol metabolites were readily formed and recovered following perfusion of rat livers with allylbenzene or estragole. We estimate that epoxidation in this experimental system accounts for approximately 20–30% of the total observed metabolism of the parent compounds. We conclude, therefore, that the epoxidation pathway is quantitatively equivalent to the 1′ hydroxylation pathway in terms of the overall metabolism of these compounds.

Our studies not only provide evidence against the first two proposed hypotheses, but they also provide clear evidence that directly supports the third hypothesis. When sub-millimolar concentrations of epoxides were added to the liver perfusion system, uptake and conversion to dihydrodiol and glutathione conjugate were almost instantaneous. This reflects the kinetics of epoxide hydrolysis observed in vitro (Table 1), whereby the K_m for the epoxides is in the low millimolar range, and the V_{max} corresponds to slightly less than 1 μmol of epoxide hydrolyzed per min per g of liver. At this turnover rate, epoxide would be metabolized much more rapidly than it is formed, and essentially no accumulation of epoxide metabolite would occur. Furthermore, a second epoxide detoxification pathway, glutathione conjugation, also exists. The rate of conjugate formation is lower than, but similar to, that of epoxide hydrolysis, and therefore glutathione conjugation represents an extra 'margin of safety', that can provide protection against these epoxides under circumstances where epoxide hydrolase activity might be impaired. At tenfold higher concentrations of epoxide, efficient detoxification is still observed. Detectable levels of epoxide are recovered during the first 20 min of perfusion, because at this high substrate concentration, epoxide hydrolases are saturated and are operating at zero-order kinetics. As the epoxide substrate concentration drops to K_m or sub-K_m values, it rapidly disappears by first-order kinetics.

Cytotoxicity studies (Fig. 8 and Table 3) also support the conclusion that the lack of toxicity of epoxide metabolites is due to their rapid detoxification rather than to their lack of reactivity or absence of formation in vivo. Although ABO and ESO are capable of forming covalent adducts in vitro, they are not directly cytosolic when per-

fused, even at very high concentrations. Although small amounts of covalent adducts to proteins are recoverable (Table 3), no DNA adducts are observed. The cytotoxicity seen when the parent compounds, allylbenzene or estragole, are perfused (Fig. 8), is therefore attributable to metabolic intermediates other than epoxide metabolites, most logically 1′ hydroxy derivatives. All evidence obtained with the perfused rat-liver model indicates, therefore, that epoxides are formed in vivo and are potentially reactive, but that they are never permitted to accumulate to levels of toxic significance due to extremely efficient detoxification by a combination of epoxide hydrolase(s) and glutathione transferase(s).

Finally, we attempted to project the significance of these findings to human exposure to natural product analogs of allylbenzene. Do these results indicate that epoxidation of these compounds represents a biotransformation pathway that results in the formation of a chemical species that binds covalently to DNA, yet is innocuous in a practical sense to humans? Our evidence suggests that this is the case. Epoxide hydrolase activity in human liver is subject to a wide variation. At least a 20-fold interindividual spread is seen between the highest and lowest observed activities (Guenthner et al., 1993). However, even those human-liver specimens with the lowest styrene oxide hydrolase activity exhibit epoxide hydrolase activity equivalent to the mean value seen with rat liver. Therefore, the degree of protection seen with the isolated perfused rat liver applies to even those human individuals with the lowest epoxide hydrolase activity, and those with average or higher activities possess an even greater margin of safety.

The concentrations of parent compounds used here are greatly in excess (at least 100-fold) of maximal exposure to eugenol, estragole, or safrole in the human diet (Proceedings, Flavor and Extract Manufacturer's Association Workshop, May 2000, in press). Furthermore, the levels of epoxides tested are in even greater than 100-fold excess compared to those levels formed in vivo after exposure to parent compounds in the diet (Proceedings, Flavor and Extract Manufacturer's Association Workshop, May 2000, in press). Therefore, we conclude that the genotoxic danger to humans of epoxide metabolites formed after dietary exposure to allylbenzene analogs is practically non-existent. We consider this to be an excellent example of the ''threshold phenomenon'' for a reactive intermediate. This intermediate, the allylic epoxide metabolite of these naturally occurring allylbenzene analogs, is highly electrophilic and readily forms covalent adducts with DNA bases in vitro. These adducts are formed at a similar rate to the rates of formation of covalent DNA adducts with styrene oxide, or aflatoxin B1, for example (Essigmann et al., 1977; Margison and Pegg, 1981; Hemminki and Hesso, 1984). However, the presence of multiple deactivation enzymes, present in humans at relatively very high levels, insures that these intermediates never accumulate in human liver, even in those individuals whose epoxide hydrolase activity places them at the low end of the interindividual spectrum. Potential genotoxicity predicted by reactivity in vitro does not translate to practical genotoxicity in vivo, and there does appear to be a relatively high 'threshhold level' for generation of these reactive epoxide metabolites, below which no practical genotoxic hazard exists.

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