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Human Cytochrome P450 Enzyme Specificity for Bioactivation of Safrole to the Proximate Carcinogen 1′-Hydroxysafrole

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In the present study, the cytochrome P450 mediated bioactivation of safrole to its proximate carcinogenic metabolite, 1′-hydroxysafrole, has been investigated for the purpose of identifying the human P450 enzymes involved. The 1′-hydroxylation of safrole was characterized in a variety of in vitro test systems, including Supersomes, expressing individual human P450 enzymes to a high level, and microsomes derived from cell lines expressing individual human P450 enzymes to a lower, average human liver level. Additionally, a correlation study was performed, in which safrole was incubated with a series of 15 human liver microsomes, and the 1′-hydroxylation rates obtained were correlated with the activities of these microsomes toward specific substrates for nine different isoenzymes. To complete the study, a final experiment was performed in which pooled human liver microsomes were incubated with safrole in the presence and absence of coumarin, a selective P450 2A6 substrate. On the basis of the results of these experiments, important roles for P450 2C9*1, P450 2A6, P450 2D6*1, and P450 2E1 were elucidated. The possible consequences of these results for the effects of genetic polymorphisms and life style factors on the bioactivation of safrole are discussed. Polymorphisms in P450 2C9, P450 2A6, and P450 2D6, leading to poor metabolizer phenotypes, may reduce the relative risk on the harmful effects of safrole, whereas life style factors, such as the use of alcohol, an inducer of P450 2E1, and barbiturates, inducers of P450 2C9, and polymorphisms in P450 2D6 and P450 2A6, leading to ultraextensive metabolizer phenotypes, may increase the relative risk.

Introduction

Alkenylbenzenes such as safrole, methyleugenol, and estragole are important constituents of herbs such as nutmeg, cinnamon, anise star, tarragon, sweet basil, sweet fennel, and anise vert. Recently, the EU Scientific Committee on Food (EU-SCF) launched scientific evaluations on these three alkenylbenzenes (*1*−*3*). The EU-SCF concluded that safrole, methyleugenol, and estragole are genotoxic and carcinogenic and indicated restrictions in use. Methyleugenol and estragole are used as flavoring agents in a variety of consumer dietary products (baked goods, nonalcoholic beverages, condiments, ice cream, and chewing gum, as well as hard and soft candies). Safrole, however, has already been banned as an additive in food by the U.S. Food and Drug Administration (Federal Register of December 3, 1960, 25 FR 12412) and by the Council of the European Communities (*4*).

In contrast to the EU-SCF panel, an industrial expert panel from the Flavor and Extract Manufacturers Association (FEMA) published that exposure to methyleugenol and estragole, resulting from spice consumption, does not pose a significant cancer risk for humans (*5*). These opposite expert judgments mainly result from a general problem in risk assessment studies. This is the absence of adequate scientific data to support unequivocal translation of carcinogenicity data of rodent animal experiments to the human situation.

One of the important issues that needs to be solved to enable better judgment of the risk posed by these alkenylbenzenes, is the identification of the cytochrome P450 enzymes involved in the bioactivation of these alkenylbenzenes to their proximate carcinogenic 1′-hydroxy metabolites.

Some data from animal experiments exist, especially for methyleugenol (*6*), showing that phenobarbitone, isosafrole, and dexamethasone induced rat liver microsomes are especially active in the 1′-hydroxylation

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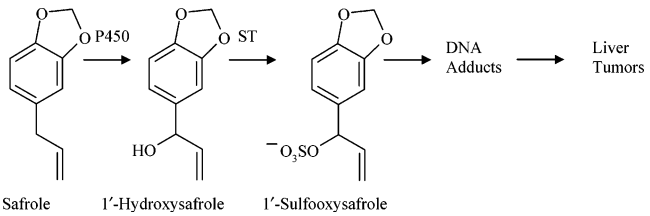

Figure 1. Bioactivation of safrole (P450 = cytochrome P450 enzymes; ST = sulfotransferase enzymes).

of this alkenylbenzene. In combination with the outcomes of studies with specific inhibitors for individual P450 enzymes, the authors suggested P450 2E1 and another enzyme, most probably P450 2C6, to be the P450 enzymes involved, whereas they excluded P450 3A, 1A2, 2D1, and 2C11 from being involved.

However, more specific studies seem to be required to unequivocally identify the P450 enzymes responsible for the 1′-hydroxylation of these alkenylbenzenes. In the present study, safrole was chosen as the model alkenylbenzene and human microsomal preparations were used. The aim of the present study was to identify the human P450 enzymes responsible for the conversion of safrole to its proximate carcinogenic metabolite 1′-hydroxysafrole. 1′-Hydroxylation of safrole is believed to be the first step in the major bioactivation pathway giving rise to the genotoxicity of safrole (*7*−*9*). This pathway, which is the same as the main bioactivation route for the structurally related alkenylbenzenes methyleugenol and estragole, is shown in Figure 1 and consists of P450 dependent 1′-hydroxylation of safrole, followed by the conversion to 1′-sulfooxysafrole by sulfotransferase enzymes. Cleavage of the sulfate moiety will lead to an electrophilic carbocation, which can covalently bind to macromolecules and cause DNA damage (*7*−*9*).

In the present study, the role in the 1′-hydroxylation of safrole of the individual P450 enzymes P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, which are the most important isoenzymes, present in the human liver and involved in drug and xenobiotics metabolism, was studied. Safrole was incubated with Supersomes, expressing these enzymes to a high level, and Gentest microsomes, prepared from lymphoblast cell lines expressing the same enzymes to an average liver level. Additionally, safrole was incubated with microsomes obtained from 15 individual human livers. The rates of 1′-hydroxylation found in these microsomes were correlated with the activities toward specific substrates for each isoenzyme. The results obtained with the Supersomes will give information about the intrinsic capacity of each P450 enzyme to 1′-hydroxylate safrole. The results from the incubations with Gentest microsomes and the correlation experiment will give information on the contribution of each individual P450 enzyme in the 1′-hydroxylation of safrole, taking into account the relative concentrations of the enzymes in the liver. The data of these experiments are especially required to enable a better definition of genetic human polymorphisms, but also life style factors that may influence human cancer risks as a result of exposure to safrole.

Materials and Methods

Materials. Chemicals. $MgCl_2 \cdot 6H_2O$, ascorbic acid, and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid and ammonium chloride (NH_4Cl) were from Acros (Geel, Belgium). Glucose-6-phosphate, $NADP^+$, and NADPH were obtained from Boehringer (Mannheim, Germany). Glucose-6-phosphate dehydrogenase and HCl (37%) were purchased from Roche Diagnostics (Mannheim, Germany). Safrole and coumarin were obtained from Sigma (St Louis, MO). Tris(hydroxymethyl)aminomethane was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were high performance liquid chromatography (HPLC) grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Piperonal, vinylmagnesium bromide (1 M solution in THF), diethyl ether, magnesium sulfate, and tetrahydrofuran (THF) were purchased from Aldrich Chemie (Steinheim, Germany). All other chemicals were from the highest quality available.

Microsomal Preparations. Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes P450 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4, and Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes, were obtained from BD Gentest (Woburn, MA). In Gentest microsomes, the activities toward enzyme-selective substrates, expressed as $pmol \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$, are on the same order as the mean activities found in human liver microsomes. In Supersomes, the enzymes are expressed to a much higher level than in the human liver (described in the Gentest Catalog). Microsomes from 15 individual human livers were obtained from Human Biologics (Phoenix, AZ). Pooled human liver microsomes were purchased from BD Gentest (Woburn, MA).

Methods. Synthesis of 1′-Hydroxysafrole. 1′-Hydroxysafrole was synthesized on the basis of the method developed by Tamayo and Ossorio (*10*) and adapted by Suga et al. (*11*) and Borchert et al. (*12*). In short, 1′-hydroxysafrole was synthesized via the Grignard reaction, starting from piperonal and vinylmagnesium bromide (Grignard reagent, 1 M solution in THF). The two reagents were used in a molar ratio of 0.5 and the reaction took place under anhydrous conditions in a nitrogen atmosphere. In detail, 0.0165 mol of piperonal was dissolved in 10 mL of dry THF and the solution was added dropwise over a period of 30 min, with stirring at 50 °C, to 0.035 mol of the Grignard reagent. The reaction was allowed to continue for another 90 min and was then poured into a solution of 4.5 g of ammonium chloride in 200 mL of ice water. The emulsion was stirred for several minutes and 1′-hydroxysafrole was extracted with diethyl ether. The organic solution was dried over magnesium sulfate and the desired compound was isolated. Structural confirmation was acquired by the UV (λ_{max} 238 and 286 nm) and MS data [m/z = 178 (M^+), 161 ($M^+ - OH$), 149, 135, 131] of the compound (*12*). The purity of 1′-hydroxysafrole was more than 98%, according to GC and HPLC analysis.

In Vitro Incubations. 1. Microsomal Incubations Using Recombinant Enzymes. Incubations with Supersomes were performed in a final volume of 100 μL and an NADPH-generating system was used. The NADPH-generating system [1 mM $NADP^+$, 10 mM glucose-6-phosphate and 0.05 μg/mL glucose-6-phosphate dehydrogenase in 0.2 M Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$] was preincubated during 10 min at 37 °C. After 9 min, ice-cold Supersomes (0.3 nmol/mL) were added. After 10 min preincubation, the reaction was started by adding safrole (500 μM final concentration, added from a 100× concentrated stock solution in DMSO) and ascorbic acid (1 mM, to prevent any auto-oxidation of safrole). The reaction was terminated after 20 min by adding 100 μL of ice-cold methanol. Incubations were performed in triplicate. Microsomal incubations with safrole, using Gentest microsomes expressing one single P450 enzyme, were performed in a 200 μL incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 1 mg/mL microsomes in 0.2 M Tris-HCl, pH 7.4. The reaction was started by adding the substrate safrole [dissolved in DMSO (final concentration DMSO 1%)] and was performed at 37 °C. The substrate concentrations used were 500 and 200 μM (as indicated in the Results section). The reaction

was terminated after 20 min by adding 50 μL of ice-cold acetonitrile. For incubations with Gentest microsomes expressing P450 2E1, the procedure was modified. Since organic solvents inhibit P450 2E1, for this incubation, safrole was dissolved in methanol, and methanol was evaporated prior to the addition of the reaction mixture. In this case, the reaction was started by adding NADPH. Incubations were performed in duplicate or triplicate (as indicated in the Results section). For both types of microsomal preparations, control incubations without NADPH or microsomes were performed and all samples were centrifuged during 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

2. Correlation Study. The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4′-hydroxylase, S-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, and testosterone 6β-hydroxylase activities, as described previously (*13*−*15*). Paclitaxel 6α-hydroxylation was determined on the basis of the instructions on the Gentest P450 2C8 data sheet (See http://www.Gentest.com/tech_resources/ass_pac.shtm). Data on protein and P450 content were provided by the supplier. Incubations with the human liver microsomes from Human Biologics were identical to the incubations with Gentest microsomes described above. These incubations were performed in duplicate.

3. Inhibition Experiment. To investigate the role of P450 2A6 in the 1′-hydroxylation of safrole in more detail, pooled human liver microsomes were incubated with safrole, in the presence and absence of coumarin, a specific substrate for P450 2A6, also used to measure P450 2A6 activity in the human liver microsomes. The incubation was similar to the incubation with the Gentest microsomes described above, but the microsomes were preincubated for 5 min with 25 μM (final concentration) coumarin, dissolved in methanol (final concentration 1%) or with 1% methanol (controls). After 5 min, safrole (final concentration 500 μM) was added, and the mixture was incubated for 20 min. The reaction was terminated by adding 50 μL of ice-cold acetonitrile. Samples were centrifuged (2750*g*, 5 min) and stored at −20 °C until analysis.

4. Kinetic Studies Using Gentest Microsomes Expressing P450 2A6. For Gentest microsomes expressing P450 2A6, the K_m and V_{max} values were determined by incubating these microsomes with substrate concentrations ranging from 50 to 500 μM (in triplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. The data were fitted to the standard Michaelis−Menten equation $v = V_{max}/(1 + (K_m/[S]))$, where [S] = substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL information systems, Inc.)

Sample Analyses. 1. HPLC Analysis of 1′-Hydroxysafrole. Aliquots (10 μL) of each sample were analyzed using HPLC [Waters M600 liquid chromatography system, equipped with an Alltima C18 column, 150 × 4.6 mm (Alltech, Breda, The Netherlands)]. The gradient was made with ultrapure water containing 0.05% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 0.7 mL/min. A linear gradient from 10 to 30% (v/v) acetonitrile in water was applied during 12 min. The percentage of acetonitrile was kept at 30% (v/v) for 2 min, increased to 100% in 3 min, was kept at 100% for 2 min, lowered to 10% (v/v), and calibrated at these initial conditions for 10 min. The retention time of 1′-hydroxysafrole under these conditions was 22.5 min. Detection was carried out by a Waters 996 photodiode array detector at 280 nm. Quantification of the amount of 1′-hydroxysafrole was performed by means of a calibration curve, made using synthesized 1′-hydroxysafrole. The activities of the Supersomes and the human liver microsomes were calculated in nmol·min^{-1}·(nmol of P450)$^{-1}$ and the activities of the Gentest microsomes were calculated in nmol·min^{-1}·(mg of protein)$^{-1}$.

2. GC−MS Analysis of 1′-Hydroxysafrole. An incubation mixture of safrole with pooled human liver microsomes and a reference mixture containing safrole and synthesized 1′-hydroxysafrole in buffer were extracted with CH_2Cl_2. The organic layer was transferred into a new vial and the solution was concentrated under a stream of nitrogen. Aliquots (2 μL) of each sample were analyzed using an HP6890 gas chromatograph, equipped with a J&W DB-5 column (30 m × 0.25 mm, 0.25 μm film) and an HP5973 mass selective detector. The GC was programmed for a 45 min run with a temperature gradient from 50 to 250 °C at 5 °C/min. The inlet temperature was 260 °C, the split ratio was 15:1, and the pressure of the helium carrier gas was 12.0 psi. The mass spectrometer was run in the electron impact mode with electron energy at 70 eV with a mass range of *m*/*z* 30−550 and a source temperature of 280 °C.

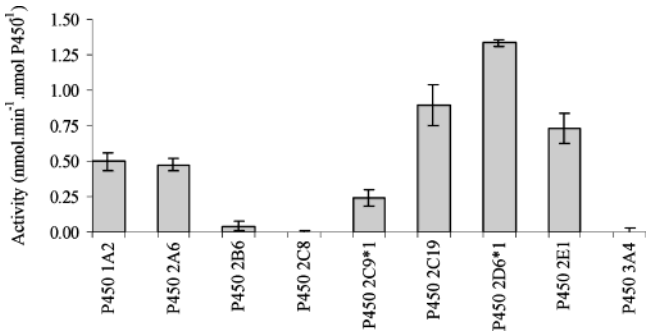

Figure 2. Safrole 1′-hydroxylation activity by Supersomes. Bars indicate average activities of triplicate measurements ± SD.

3. Pearson Correlation. In cases where enzymatic activities are expressed as pmol·min^{-1}·(nmol of P450)$^{-1}$, correlation analysis will be independent of the amount of P450 present in the various samples. Thus, for correlation between the metabolism of safrole and the P450 marker substrates, enzyme activities expressed as pmol·min^{-1}·(nmol of P450)$^{-1}$ have been used. Statistical significance was determined by the Pearson correlation test using the SAS statistical software (SAS Institute Inc., Cary, NC, Software Release 8.2).

Results

Formation of 1′-Hydroxysafrole by Recombinant P450 Enzymes. HPLC analysis of a typical incubation of pooled human liver microsomes with safrole revealed formation of one metabolite, which could be identified as 1′-hydroxysafrole on the basis of the similarity of its retention time, UV spectrum, and mass spectrum to those of the chemically synthesized reference compound and the data from the literature (*12*). To define the human P450 enzymes capable of safrole 1′-hydroxylation, incubations with Supersomes were performed. The results obtained with the Supersomes (Figure 2) clearly show that many P450 enzymes are intrinsically able to catalyze the bioactivation of safrole. Of the nine isoenzymes tested, P450 1A2, 2A6, 2C9*1, 2C19, 2D6*1, and 2E1 were able to 1′-hydroxylate safrole. Only P450 2B6, 2C8, and 3A4 showed (almost) no 1′-hydroxylation activity. Data from the incubations with Gentest microsomes are shown in Figure 3. Since in Gentest microsomes, the activities toward enzyme-selective substrates, expressed as pmol·min^{-1}·(mg of protein)$^{-1}$, are on the same order as the mean activities found in human liver microsomes, these data give an estimate of the relative contribution of each isoenzyme in the human liver. From these data, P450 2A6, 2C9*1, 2C19, 2D6*1, and 2E1 appear to be the most active isoenzymes in safrole 1′-hydroxylation at a substrate concentration of 500 μM. In contrast to the activities found for the Supersomes, P450 1A2 activity found in this experiment was very low, indicating that

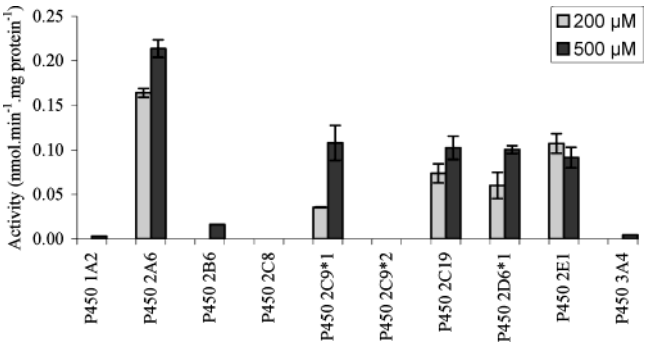

Figure 3. Safrole 1′-hydroxylation activity by Gentest microsomes at two different substrate concentrations. Bars indicate average activities of duplicate measurements (P450 1A2, 2B6, 2C8, 2C9*2, and 3A4) or triplicate measurements ± SD (P450 2A6, 2C9*1, 2C19, 2D6*1, and 2E1).

Table 1. Correlations among the Activities of 15 Human Liver Microsomal Samples toward P450 Enzyme Selective Substrates and the Formation of 1′-Hydroxysafrole by These Samples

marker substrate[a]	mean activity ± SD [pmol·min^{-1}·(nmol of P450)$^{-1}$]	P450 enzyme	correlation coefficient
EROD	149 ± 78 (22−299)	P450 1A2	0.07
COUM	3187 ± 2638 (13−10439)	P450 2A6	0.06
7-ETC	699 ± 230 (274−1122)	P450 2B6	0.09
PACL	550 ± 215 (179−905)	P450 2C8	0.18
DICLF	5789 ± 2818 (2499−12101)	P450 2C9	0.66[b]
MEPH	33 ± 57 (3−228)	P450 2C19	0.34
BUFU	378 ± 330 (52−1216)	P450 2D6	0.44
CLZOX	6409 ± 4131 (1741−14992)	P450 2E1	0.44
TEST	10497 ± 5158 (2852−20725)	P450 3A	−0.32

[a] EROD = 7-ethoxyresorufin *O*-dealkylation; COUM = coumarin 7-hydroxylation; 7-ETC =7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation; PACL = paclitaxel 6α-hydroxylation; DICLF = diclofenac 4′-hydroxylation, MEPH = *S*-mephenytoin 4′-hydroxylation; BUFU = bufuralol 1′-hydroxylation; CLZOX = chlorzoxazone 6-hydroxylation; TEST = testosterone 6β-hydroxylation. [b] Statistical significance: $P < 0.01$.

the contribution of this enzyme to safrole 1′-hydroxylation is negligible at average liver levels. For the enzymes most active in safrole 1′-hydroxylation, additional experiments were performed at a substrate concentration of 200 μM instead of 500 μM. P450 2E1 was saturated at the 200−500 μM safrole concentration range, whereas the activities of P450 2A6, 2C9*1, 2C19, and 2D6*1 were respectively 22, 68, 26, and 40% lower at a concentration of 200 μM compared to 500 μM safrole (Figure 3).

Formation of 1′-Hydroxysafrole by Human Liver Microsomes. The average rate of 1′-hydroxylation of safrole in liver microsomes from 15 individuals was 1.32 ± 0.33 nmol 1′-hydroxysafrole·min^{-1}·(nmol of P450)$^{-1}$. A 2.5-fold variation between different human liver samples was found [range: 0.77−1.95 nmol·min^{-1}·(nmol of P450)$^{-1}$]. Table 1 shows the mean ± SD activities of the panel of 15 human liver microsomes toward specific substrates for nine different isoenzymes. The calculated correlation coefficients between 1′-hydroxylation of safrole and the activity toward all the specific substrates are also given in Table 1. A significant ($P < 0.01$) correlation between P450 2C9 activity and 1′-hydroxylation of safrole was found ($r = 0.66$). The *P*-values for the correlation between the activities of the microsomes expressing P450 2D6 ($r = 0.44$) and P450 2E1 ($r = 0.44$) toward their specific substrates and the 1′-hydroxylation of safrole were 0.1, indicating that these enzymes might also play a role in the bioactivation of safrole. P450 2A6 activity showed no correlation with the 1′-hydroxylation of safrole. This result is not in agreement with the results from the Gentest microsomes, where P450 2A6 showed the highest 1′-hydroxylation activity of all isoenzymes tested (Figure 3).

Inhibition Experiment. To investigate the role of P450 2A6 in the 1′-hydroxylation of safrole in more detail, safrole was incubated with pooled human liver microsomes in the presence and absence of coumarin. An activity of 1.21 nmol 1′-hydroxysafrole·min^{-1}·(mg of protein)$^{-1}$ was found in the absence of coumarin. However, in the presence of coumarin, a specific P450 2A6 substrate, an activity of 0.68 nmol 1′-hydroxysafrole·min^{-1}·(mg of protein)$^{-1}$ was found. Thus, 1′-hydroxylation of safrole in human liver microsomes was inhibited by 44% in the presence of coumarin.

K_m and V_{max} Determination for Safrole 1′-Hydroxylation. Because Gentest microsomes expressing individual P450 isoenzymes reflect the situation in the average human liver most, and P450 2A6 appears to be the most active isoenzyme for safrole 1′-hydroxylation in this in vitro model, Gentest microsomes expressing P450 2A6 were used to determine the apparent K_m and V_{max} for the P450 2A6 mediated safrole 1′-hydroxylation. The Michaelis−Menten plot of the incubations with these microsomes revealed an apparent V_{max} of 0.22 ± 0.01 nmol 1′-hydroxysafrole·min^{-1}·(mg of protein)$^{-1}$, and an apparent K_m of 80 ± 15 μM.

Discussion

In the present study, the human cytochrome P450 enzymes involved in the bioactivation of safrole to its proximate carcinogen 1′-hydroxysafrole were studied, making use of different in vitro test systems. Four different kinds of experiments were undertaken to identify the human cytochrome P450 enzymes involved. Supersomes are a useful tool to obtain information about which P450 isoenzymes can metabolize safrole, because Supersomes have a high activity of specific isoenzymes. The results from this experiment clearly show that various cytochrome P450 enzymes are able to 1′-hydroxylate safrole. Since on the basis of these results only three P450 isoenzymes (P450 2B6, 2C8, and 3A4) are excluded from being involved in the bioactivation of safrole, all isoenzymes were also tested in the experiment with the Gentest microsomes and the correlation study. In addition to the intrinsic activity of the various P450s, these in vitro model systems also take into account the relative amount of the various P450 enzymes present in the human liver. On the basis of the results from those two experiments, an important role for P450 2C9*1 was revealed. A significant correlation ($P < 0.01$) was found between safrole 1′-hydroxylation activity and diclofenac 4′-hydroxylation activity. In the experiment with the Gentest microsomes, P450 2C9*1 showed the second highest safrole 1′-hydroxylation activity. P450 2C9 is the main enzyme of the P450 2C subfamily in the human liver. The P450 2C subfamily accounts for 20% of the total P450 content in the human liver and is, after P450 3A (almost 30%), the most abundant P450 family (*16*). However, the correlation coefficient obtained for P450 2C9 activity (0.66) does not approach 1.00. Obviously, P450 2C9 is not the only isoenzyme involved. The results from the correlation study indicate that P450 2D6 ($r = 0.44$) and P450 2E1 ($r = 0.44$) might also add substan-

tially to total safrole 1′-hydroxylation in the human liver. Moreover, the Gentest microsomes expressing those two enzymes also showed high safrole 1′-hydroxylation activity. These two enzymes are less prevalent in the human liver than P450 2C9. According to Shimada et al., P450 2D6 accounts for 1.5% and P450 2E1 for 7% of the total P450 content (*16*). Gentest microsomes expressing P450 2A6 showed the highest activity [0.22 nmol 1′-hydroxysafrole·min^{-1}·(mg of protein)$^{-1}$] of all microsomes tested, although no correlation between 1′-hydroxylation of safrole and coumarin 7-hydroxylation was observed. But since 44% inhibition of safrole 1′-hydroxylation activity was found in the P450 2A6 inhibition experiment, using pooled human liver microsomes and the specific P450 2A6 substrate coumarin, it is clear that P450 2A6 also plays an important role in the bioactivation of safrole. In human liver, P450 2A6 is a minor enzyme; it accounts for approximately 4% of the total P450 content (*16*), but it is known to be involved in the bioactivation of various precarcinogens, like aflatoxin B_1 and *N*-nitrosamines (*17*). In the human liver microsomes used in the correlation study, an 800-fold difference in coumarin 7-hydroxylation is present [range 13−10493 pmol·min^{-1}·(nmol of P450)$^{-1}$]. For the other isoenzymes, the differences in activity in the 15 human liver samples are much smaller, ranging between 4- and 76-fold. This, in combination with the low abundance of P450 2A6 in the human liver (4%), might explain the disagreement between the results with the Gentest microsomes and the inhibition study for P450 2A6 and the correlation study. For P450 2A6, an apparent K_m of 80 ± 15 μM was revealed. For P450 2C9*1, 2D6*1, and 2E1, no K_m and V_{max} determination were performed, but additional incubations at 200 μM substrate concentration indicated P450 2E1 to be saturated at the 200−500 μM safrole concentration range, whereas the activities of P450 2A6, 2C9*1, and 2D6*1 were respectively 22, 68, and 40% lower at a safrole concentration of 200 μM compared to 500 μM. Therefore, the relative contribution of P450 2E1 to safrole 1′-hydroxylation at lower concentrations might be even higher than estimated from the in vitro experiments at 500 μM safrole concentration. However, at 200 μM substrate concentration, P450 2A6 still appears to be the most active isoenzyme in safrole 1′-hydroxylation.

Taken together, the results obtained in the present experiments indicate P450 2C9*1, 2A6, 2D6*1, and 2E1 to be the main isoenzymes involved in the bioactivation pathway of safrole to the proximate carcinogen 1′-hydroxysafrole in man.

Gardner et al. have published a paper about the isoenzymes involved in 1′-hydroxylation of a related alkenylbenzene, methyleugenol, investigated with rat liver microsomal model systems (*6*). The authors suggested P450 2E1 and another enzyme, most probably P450 2C6, to be one of the most important isoenzymes for this bioactivation. These experiments were conducted with rat liver microsomes, so P450 2A6 and P450 2C9*1 were not tested. Therefore, it would be interesting to also investigate with different in vitro systems whether the same human P450 enzymes are involved in the bioactivation of the related alkenylbenzenes methyleugenol and estragole. Interestingly, Gardner et al. found a 37-fold variation in the methyleugenol 1′-hydroxylation activity (substrate concentration 200 μM) in 13 human liver microsomes. In our study, we found a 2.5-fold difference in safrole 1′-hydroxylation activity in the 15 human liver microsomes used in the correlation study. This suggests that for methyleugenol, interindividual differences may be even more important for the risk assessment than for safrole. Interindividual variation in the bioactivation of safrole is probably caused in part by polymorphisms in the P450 enzymes involved. This could lead to "poor metabolizer" and "ultrarapid metabolizer" phenotypes. In the experiments with the Gentest microsomes, P450 2C9*2 was also tested besides the wild-type enzyme P450 2C9*1. Although the wild-type enzyme P450 2C9*1 showed a high safrole 1′-hydroxylation activity, P450 2C9*2 did not show any measurable safrole 1′-hydroxylation. P450 2C9*2 results from the ^{416}P450 2C9 C >T mutation, causing a change of ^{144}Arg to Cys. Besides the mutated allele P450 2C9*2, another variant, P450 2C9*3, with a ^{359}Iso to Leu change, has been identified. The frequencies of the alleles P450 2C9*1, P450 2C9*2, and P450 2C9*3 in the Caucasian population are 0.86, 0.08, and 0.06, respectively (*18*).

Besides P450 2C9, also P450 2A6 and P450 2D6 are polymorphic (for a review, see ref *19*). There is a marked interindividual variation in coumarin-7-hydroxylation activity in humans due to a genetic polymorphism that exists in the human P450 2A6 gene. At least six allelic variants have been identified in addition to the wild-type allele (*17*). Population studies suggest that 6% of the UK population is homozygous for mutant P450 2A6 alleles, whereas in Japanese subjects the mutant allele frequency may be as high as 48% (*20*, *21*). P450 2A6 polymorphism has been investigated extensively because especially the P450 2A6 whole deletion phenotype is believed to be related to a decreased risk for lung cancer, due to decreased nicotine metabolism or decreased bioactivation of *N*-nitrosamines from tobacco smoke (*17*, *22*, *23*).

P450 2D6 is a highly polymorphic P450 isoform. At least 41 different alleles of P450 2D6 are known, leading to poor but also to increased enzyme activities (*24*). According to Meyer (*25*), 5.0−13.0% of the Caucasian population has a P450 2D6 genotype leading to a poor metabolizer phenotype. In the same population, 1.0−10.0% are ultrarapid metabolizers due to P450 2D6 gene duplication or multiplication (*25*). For a risk assessment for the use of safrole, this last category of ultrarapid metabolizers is the most important one, possibly being at higher risk for the harmful effects of safrole if exposure occurs. Besides polymorphisms as causes for interindividual differences in safrole 1′-hydroxylation, also lifestyle factors might influence one's susceptibility for safrole genotoxicity. P450 2E1 is induced in both rats and humans by ethanol (*26*, *27*). Therefore, a wide variety in P450 2E1 levels will be found in the human population. This implies that people who consume more alcoholic beverages might be at higher risk from exposure to precarcinogens bioactivated by P450 P2E1. The same might be true for people who use barbiturates, which induce, among others, P450 2C enzymes (*28*, *29*). In conclusion, our results suggest that P450 2C9*1, 2A6, 2D6*1, and 2E1 are the most important isoenzymes in the bioactivation of safrole. For a risk assessment for the adverse effects of safrole, special attention has to be paid to groups at higher risk in the population, due to the excessive use of alcohol or barbiturates or polymorphisms in especially the P450 2D6 gene leading to ultrarapid metabolizer phenotypes.

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Note Added after ASAP Posting. In the first paragraph of the section titled Sample Analyses, the unit of Gentest microsome activities was incorrectly posted ASAP August 25, 2004; the corrected version was posted August 30, 2004.

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