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THE METABOLISM OF PHENYTOIN
BY HUMAN LIVER MICROSONES AND CYTOCHROME P450S
EXPRESSED IN SACCHAROMYCES CEREVISIAE
AND COS-1 CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the
Graduate School of The Ohio State University

By

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1995

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DEDICATION

To Mary Ellen and our families
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PUBLICATIONS


**ABSTRACTS**


FIELDS OF STUDY

Major field: Pharmacology
Minor fields: Drug Metabolism
Molecular Biology
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LIST OF ABBREVIATIONS

ACN  acetonitrile
BCA  bichicinnoic acid
CAT  catechol metabolite
CIAP  calf intestinal alkaline phosphatase
DHD  dihydrodiol metabolite
DLPC  dilaurylphosphatidylcholine
DMSO  dimethyl sulfoxide
DTT  dithiothreitol
DPH  diphenylhydantoin, phenytoin
EDTA  ethyldiaminetetraacetic acid
HEPES  (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid])
HPLC  high performance liquid chromatography
HPPH  hydroxyphenylphenylhydantoin
MeOH  methanol
NADP  nicotinamide adenine dinucleotide phosphate
NADPH  nicotinamide adenine dinucleotide phosphate, reduced form
PCR  polymerase chain reaction
PEG  polyethylene glycol
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE  Tris-acetate-EDTA
TE  Tris-EDTA
TBE  Tris-borate-EDTA
TEMED  N, N, N’, N’-tetramethylethylenediamine
YPD  yeast peptone broth with dextrose
INTRODUCTION

Clinical use of DPH:
In 1938 Merritt and Putnam, following a systematic search for compounds which modify the electroshock threshold, described the anticonvulsant properties of phenytoin (DPH, 5,5-diphenylhydantoin, Dilantin) (1,2). The first anticonvulsant without significant sedative properties at therapeutic concentrations, it is active not only in partial and generalized seizures, but also in status epilepticus. DPH decreases the rate of depolarization of abnormal neuronal cells by use-dependent blockage of voltage-gated sodium channels (3-5) and also blocks the influx of calcium through voltage-gated calcium channels (6-8). This blockage of channels limits the spread of the electrical impulse. In the heart, DPH slows conduction through the atria, increases AV nodal conduction and attenuates digitalis-induced ventricular and supraventricular arrhythmias (7, 9, 10).

Pharmacokinetics and Toxicity of DPH:
The usual dose of DPH is 300 mg/day and therapeutic levels in plasma are 10-20 μg/ml. Dose-related side effects occur as concentrations in plasma exceed 20 μg/ml and include cerebellar ataxia, tremor, nystagmus, diplopia,
and vertigo. Chronic administration of DPH can cause gingival hyperplasia, hirsutism, peripheral neuropathy, megaloblastic anemia, and hypersensitivity reactions (11, 12). The latter hypersensitivity reactions may be characterized by fever, skin rash, lymphadenopathy and/or hepatitis. It has been postulated that these reactions may be caused by immune responses to macromolecules covalently bound to highly reactive metabolites of DPH (13, 14). Spielberg et al. and others have suggested that hypersensitivity reactions to DPH are correlated with the capacity of patients to detoxify reactive metabolites of DPH. Patients less able to detoxify reactive products were more prone to hypersensitivity. This was determined by measuring the cytotoxicity of DPH to lymphocytes from these patients in incubations of the drug with liver microsomes of mice. Patients with higher cytotoxicity had an increased incidence of DPH related toxicity (15-20).

Infants of mothers exposed to DPH during pregnancy may develop the fetal hydantoin syndrome, characterized by cleft lip and/or palate, cardiovascular anomalies, renal and limb deformities, diaphragmatic hernia, hypoplastic nails and growth retardation (21-3). The teratogenic effect, confirmed by animal studies, has been attributed to 1 of 3 factors: 1) decreased metabolism of folic acid (24, 25); 2) chemically reactive products, such as epoxides, formed by cytochrome P450 enzymes during the metabolism of DPH, which bind to fetal tissues and DNA (26-32); or 3) free radicals of DPH, formed during the
oxidation of arachidonic acid by cyclooxygenase or lipoxygenase, which bind to fetal tissues and DNA (33-8). Beuhler et. al. have provided evidence that the fetal hydantoin syndrome is correlated to the activity of microsomal epoxide hydrase in amniocytes (39). These studies, over many years, suggest that the metabolism of DPH may be responsible for some of the clinical toxicity.

The elimination of DPH from plasma of humans at concentrations below 10 μg/ml approximates first-order kinetics; however, the elimination at concentrations greater than 20 μg/ml is nonlinear and approaches a zero-order process. The nonlinear elimination of DPH in humans (40, 41), rats (42), mice (43), and dogs (44) is probably caused by saturation of the microsomal cytochrome P450 enzymes in the liver which convert DPH to hydroxyphenylphenylhydantoin (HPPH), the major metabolite (45), and/or inhibition of these enzymes by HPPH (46, 47).

It is important to monitor the concentration of DPH in plasma because of its narrow therapeutic range and nonlinear pharmacokinetics. Approximately 5% of Caucasians eliminate DPH very slowly (40, 48, 49). This may be an inherited trait, but does not correlate with other common polymorphisms of drug metabolism such as the metabolism of debrisoquine and (S)-mephenytoin (48, 50, 51).
Metabolism of DPH to major metabolites:

In 1957 Butler showed that the major metabolite of DPH in the urine of humans and rats is \( p \)-HPPH, which was excreted as a conjugate and accounted for 70% of the administered dose (52). Maynert identified the metabolite as a glucuronide conjugate (53). This glucuronide conjugate is excreted via the bile into the intestine, where it is cleaved to \( p \)-HPPH by \( \beta \)-glucuronidase from intestinal cells and/or bacteria, and the aglycone reabsorbed into blood. The \( p \)-HPPH is reconjugated in the liver and undergoes extensive enterohepatic recirculation (42). In contrast, Atkinson discovered in 1970 that the major metabolite of DPH in dogs is the glucuronide conjugate of \( m \)-HPPH (54). Also in 1970, Chang isolated an unconjugated dihydrodiol metabolite (DHD, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin) from the urine of rats and monkeys (55). This metabolite was subsequently identified in the urine of humans (56-8), mice (59), and dogs (57). After the identification of the DHD metabolite, it was postulated that \( p \)-HPPH and DHD may be formed by cytochrome P450 enzymes through an epoxide intermediate. The hydroxylated metabolites of DPH formed by cytochrome P450 enzymes are illustrated in Figure 1.

The NIH shift was first reported in 1967 by Guroff et al. to describe the migration of deuterium from the \textit{para}- position to the \textit{meta}- position during \textit{para}- hydroxylation of aromatic substrates (60). The migration was
Figure 1. *Hydroxylated metabolites of DPH*: The major metabolite of DPH in humans is $\rho$-HPPH, which may be formed through a proposed epoxide intermediate or direct hydroxylation.
postulated to be caused by the formation of an epoxide intermediate. Other toxic and/or carcinogenic aromatic hydrocarbons, such as benzo[a]pyrene and phenanthracene, were shown to be metabolized to phenols, epoxides, and dihydrodiols, suggesting that DPH and other aromatic substrates may be metabolized through epoxide intermediates (61). Studies in vivo have examined the NIH shift during the metabolism of deuterium-substituted DPH. In rats and humans given racemic 5-(4-deuteriophenyl)-5-phenylhydantoin, 68-72% of the deuterium was retained in p-HPPH (62). Dogs administered DPH substituted with deuterium at the para-, meta-, and ortho- positions retained the radiolabel in both m- and p-HPPH (63).

Isotope effects, which compare the rate of hydroxylation of a substrate labelled with hydrogen or deuterium, have helped elucidate reaction mechanisms. If a carbon-hydrogen bond is broken during a direct hydroxylation reaction, the rate of that reaction for the substrate with a carbon-deuterium bond will be slower. For a positive isotope effect, the ratio of the rates for the two reactions (kH/kD) is greater than one. Isotope effects in the metabolism of DPH to HPPH have been measured in rats to determine whether p-HPPH is formed through an epoxide intermediate. A positive isotope effect for the metabolism of p-deuteriophenytoin or decadeuteriophenytoin to p-HPPH was not observed (64, 65), indicating that p-HPPH may be formed by an epoxide intermediate, not by insertion of the
oxygen between a carbon-hydrogen bond.

The formation of diol metabolites from epoxides of other aromatic substrates, especially carcinogenic polycyclic aromatic hydrocarbons, the NIH shift and isotope effect information with DPH, together with the discovery of the DHD metabolite lead to the hypothesis that \( p \)-HPPH and DHD are formed through a common epoxide intermediate.

The two phenyl groups on the 5 position of the hydantoin ring of DPH render the molecule prochiral. In humans, rats, and mice, DPH is hydroxylated primarily on the (S)-, whereas in dogs it is primarily on the (R)-phenyl ring.

The ratios of the stereochemical isomers of \( p \)-HPPH and DHD have been determined by HPLC, GC, and circular dichroism \((47, 58, 66-9)\). The configuration of (S)-\( p \)-HPPH and (R)-\( m \)-HPPH has been confirmed by optical and/or X-ray diffraction studies with synthetic, cyclohexyl derivatives of these metabolites \((70, 71)\). In humans, the stereochemical ratios of the DHD and \( p \)-HPPH excreted in urine are approximately 90/10 \((S)/(R)\) and 75/25 \((S)/(R)\), respectively \((57, 58, 69, 72)\). If the DHD and the \( p \)-HPPH are formed through the same epoxide intermediate, the stereochemical ratios of these two metabolites should be the same. This is clearly not the case, suggesting that the established notion of a common intermediate is questionable.
Metabolism of DPH to additional metabolites:

A catechol (CAT) and a methylated catechol (O-MeCAT) metabolite of DPH have been isolated from the urine of rats and identified by GC/MS (73, 74). Subsequently, these metabolites were identified in urine from humans (75) and mice (59). Glucuronide conjugates of both metabolites have also been identified by GC/MS analysis of permethylated and perdeuteriomethylated bile from an isolated rat liver perfused with a solution containing DPH (76, 77).

Several minor metabolites of DPH have also been identified. Thompson et al. identified 5,5-bis(4-hydroxyphenyl)hydantoin as a glucuronide conjugate in permethylated bile from rats and urine from humans (78). The latter was first proposed in 1969 by Woodbury, who characterized urinary metabolites in humans by solvent extraction (79). Another metabolite, also identified in rat bile and human urine, was a conjugate of glucuronic acid on a nitrogen of the hydantoin ring (80). The 2,2-diphenylhydantoic acid, formed by scission of the hydantoin ring, has been reported in urine from dogs and mice but not humans (59, 72).

Incubation of DPH with human neutrophils activated with phorbol esters produced p-, m-, and o-HPPH metabolites identified by GC/MS of the TMS derivatives. Additionally, extremely unstable mono- and di-N-chlorinated products and other much more polar compounds, probably produced by
Catalytic cycle of cytochrome P450 enzymes:
The catalytic cycle of cytochrome P450 enzymes is shown in Figure 2. In reactions of cytochrome P450 enzymes with \( \text{O}_2 \), NADPH, and substrate, the catalytic cycle of the enzyme begins with substrate binding to the enzyme (Figure 2, [a]). The iron in the heme prosthetic group of the enzyme is reduced to the ferrous state by an electron from NADPH-oxidoreductase (Figure 2, [b]), which enables oxygen to bind to the enzyme, forming an activated iron-oxygen complex with the resonance structure \( \text{Fe}^{+2} \text{(O}_2^\cdot) \) (Figure 2, [c]). The protonation state and the electron configuration of the activated iron-oxygen complex is not known. A second electron is transferred to the enzyme by NADPH-oxidoreductase or cytochrome \( b_5 \), which results in the release of water and hydroxylation of the substrate (Figure 2, [d]), leaving the iron in the ferric state.

It is uncertain how the enzyme transfers the oxygen from the activated iron-oxygen complex (Figure 2, [c]) to aromatic substrates. For aliphatic compounds the cytochrome P450 enzyme abstracts a hydrogen from the substrate and donates a hydroxyl radical from the activated iron-oxygen complex (83, 84). The precise mechanism by which oxygen is inserted into the phenyl ring of DPH is unclear.
Figure 2. Catalytic cycle of cytochrome P450 enzymes: The catalytic cycle of cytochrome P450 enzymes begins with substrate binding to the enzyme [a]. The iron in the heme prosthetic group of the enzyme is reduced to the ferrous state by an electron from NADPH-oxidoreductase [b], which enables oxygen to bind to the enzyme forming an activated iron-oxygen complex [c]. A second electron is transferred to the enzyme by NADPH-oxidoreductase or cytochrome b₅, which results in the release of water from the enzyme and the hydroxylation of the substrate [d], leaving the iron in the ferric state.
The extremely reactive activated iron-oxygen complex formed by the cytochrome P450 enzyme (Figure 2, [c]) can autooxidize, producing superoxides (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) which can react with the substrate to form hydroxylated products (85-7). The rate of formation of hydrogen peroxide is dependent on the rate of the transfer of the second electron to the cytochrome P450 enzyme and how efficiently the substrate binds to the cytochrome P450 enzyme (88).

Organization of cytochrome P450 enzymes:
The metabolites of DPH may be formed by a number of cytochrome P450 enzymes in the liver. The isozymes of cytochrome P450 are categorized by their gene structure (89). In humans 35 genes for cytochrome P450 enzymes have been identified. Genes encoding these isozymes are empirically divided into 62 families and 110 subfamilies. By definition, genes in the same family have greater than 40% nucleic acid homology, whereas genes in the same subfamilies have greater than 50% homology. Cytochrome P450 enzymes within a subfamily with less than 3% divergence in the amino acid sequence are defined as allelic variants of the same gene.

In humans, four cytochrome P450 isozymes have been identified in the 2C subfamily: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Although two additional CYP2C isozymes have been described, one of these, CYP2C10, is
a cloning artifact of an allelic variant of CYP2C9, and the other, CYP2C17, is a splice mutation of CYP2C18 and CYP2C19 (89). Several allelic variants have been identified for CYP2C9 (90) and for CYP2C18 (90-1), and each allelic variant of CYP2C9 has a different rate for the metabolism of warfarin, tolbutamide, and DPH (92-5).

Frequently studied substrates of cytochrome P450 enzymes in the 2C subfamily are (S)-mephenytoin, warfarin, and tolbutamide. Cytochrome P450 2C8 metabolizes (S)-mephenytoin, tolbutamide, warfarin, DPH, arachidonic acid, benzo[a]pyrene, retinol, and carbamazepine (92-6). Like cytochrome P450 2C8, cytochrome P450 2C9 metabolizes (S)-mephenytoin, tolbutamide, warfarin, DPH, arachidonic acid, and benzo[a]pyrene, but it also metabolizes omeprazole and torsemide, although not retinol or carbamazepine (90, 92-5, 98-102). Cytochrome P450 2C19 is the primary isozyme that metabolizes (S)-mephenytoin to 4'-hydroxymephenytoin (90, 97, 103, 104); it also metabolizes tolbutamide and warfarin (93, 104). Cytochrome P450 2C18 metabolizes (S)-mephenytoin, tolbutamide and warfarin (90, 93, 104).

Expression of cytochrome P450 enzymes in heterologous systems:
Several approaches have been used to study the metabolism of substrates by cytochrome P450s, including incubation of the substrate with liver microsomes, isolated cells, enzymes purified from tissues, and enzymes
expressed in heterologous expression systems. The activity of a single cytochrome P450 enzyme expressed in a heterologous system can be studied without interference from other enzymes. The first heterologous expression system for a cytochrome P450 enzyme was *Xenopus* oocytes (105), followed subsequently by COS-1 cells (106-7), *Saccharomyces cerevisiae* (108-11), *E. coli* (112-3), AHH-1 lymphoblastoid cells (114-5) and baculovirus mediated expression in *Spongifera putifera* (116).

Studies with expressed cytochrome P450 enzymes, NADPH-oxidoreductase, cytochrome b₅, and microsomal epoxide hydrase may elucidate the activities of these proteins. The significance of cytochrome b₅ for catalytic activity and the importance of microsomal epoxide hydrase for the formation of dihydriodiols have been determined by expression of these proteins in heterologous systems (117-21). Cytochrome P450 enzymes, modified by site-directed mutations, have been expressed in heterologous systems to determine which amino acids are important for catalytic activity and/or regio- and stereoselectivity of hydroxylation reactions (122-5). Further, by expressing cytochrome P450 enzymes, researchers have determined amino acids important for O₂ binding to the heme prosthetic group, substrate binding, and transfer of electrons to the cytochrome P450 enzyme by NADPH-oxidoreductase and cytochrome b₅ (126-8).
There are, however, disadvantages to studying the metabolism of substrates with expressed proteins rather than in animals or in tissue homogenates. Metabolites formed in microsomes of liver through sequential metabolism by several cytochrome P450 enzymes will only be formed in these heterologous systems only if the correct proteins are coexpressed. The amount of a cytochrome P450 enzyme formed by the expression system may be higher than \textit{in vivo} or in microsomes from liver. This may lead to the production of metabolites not usually present \textit{in vivo} or in incubations of the drug with liver microsomes.

\textbf{Background to the specific aims:}

The metabolism of DPH has been correlated with that of tolbutamide, a substrate known to be metabolized by 2C isozymes. Although, the metabolism of DPH to a hydroxylated product by cytochrome P450 2C9 expressed in COS-1 cells has been shown by Veronese \textit{et al}, the formation of other metabolites in COS-1 cells has not been studied (129, 130). In addition, the metabolism and the profile of metabolites of DPH resulting from the homologous cytochrome P450 enzymes 2C8 and 2C18 have not been characterized. These isozymes are likely to metabolize DPH, evidenced by their metabolism of tolbutamide, warfarin, and (S)-mephenytoin, although the mechanism(s) by which DPH is metabolized by isoymes of cytochrome P450 in the liver is unclear.
Specific aims:

This study was undertaken to:

i) Determine the pattern of metabolites of DPH incubated with isolated human microsomes; determine whether the pattern included o-, m-, p-HPPH, CAT, and the DHD.

ii) Determine which isozymes of cytochrome P450 produced one or more of these metabolites in vitro.

iii) Clarify the mechanisms of formation of HPPH by studying whether it was produced independently of the DHD and other hydroxylated metabolites, thereby helping determine whether an epoxide existed as a reactive intermediate in the formation of p-HPPH.

In order to accomplish these aims, it became necessary to express isozymes of cytochrome P450 in COS-1 cells and S. cerevisiae.
MATERIALS AND METHODS

Materials:
acrylamide, ammonium acetate, ammonium persulfate, ampicillin, β-glucuronidase, β-mercaptopethanol, BCIP (5-bromo-4-chloro-3-indolyl phosphate), calcium chloride, dilaurylphosphatidyl choline, dimethyl sulfoxide, dithiothreitol, DPH, EDTA, ethidium bromide, glucose, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glycerol, HEPES, isoamyl alcohol, lithium acetate, NADP, NADPH, NBT (nitro blue tetrazolium), sodium chloride, sodium hydroxide, D-sorbitol, sucrose, TEMED, Tris-Cl, Tween-20, and xylene cyanol FF were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile, n-chlorobutane, isopropanol, methanol, PEG-4000, sodium dodecyl sulfate and sodium hydroxide were purchased from Fisher Scientific Company (Cincinnati, OH). Ampicillin-streptomycin, Dulbecco’s modified Eagle media, fetal calf serum, Lennox L broth base (LB), Lipofectin, Opti-MEM, 10X phosphate buffered saline, protein markers, Select agar, Select peptone 140, Select yeast extract, and trypsin-EDTA were purchased from Gibco-BRL (Life Technologies, Gaithersburg, MD).
Methods:

Construction of 2C8- and 2C9/pcDNA:

The cDNAs encoding cytochrome P450s 2C8 and 2C9 (131) in vector pUVI (132) were a gift from Dr. Frank Gonzalez (Laboratory of Molecular Carcinogenesis, NCI, NIH). The *E. coli* containing the 2C8-pUVI or 2C9-pUVI were streaked on LB agar plates containing 100 μg/ml ampicillin (AMP) and incubated overnight at 37°C. Single colonies of *E. coli* were selected from the plates and used to inoculate 10 ml of LB broth containing 100 μg/ml ampicillin. These cultures were grown overnight in a 37°C shaking water bath. The plasmid DNA was isolated from the *E. coli* by the boiling mini-prep method and redissolved in 53 μl of water. The CYP2C8 and CYP2C9 cDNA were cut out of the pUVI vector by digestion of the mini-prep DNA with EcoRI by addition of a 25 μl portion of the mini-prep DNA to a 1.5 ml eppendorf tube containing 3 μl React 3 buffer (Gibco-BRL), and 1 μl EcoRI (Gibco-BRL, 10 U/μl). A control incubation contained 26 μl of DNA and 3 μl React 3 buffer with no restriction enzyme. The tubes containing the cDNA for cytochrome P450 2C9 were incubated for 1 hour at 37°C. Tubes containing the cDNA for cytochrome P450 2C8 were incubated at 37°C for only 5 minutes owing to the presence of an internal EcoRI restriction site in the CYP2C8 gene. RNA in the samples was degraded by adding 1 μl of 10 mg/ml RNaseA (Gibco-BRL) to each incubation, and the reactions stopped by
addition of 4 µl of 5X agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 50% glycerol in 1X TAE or 1X TBE). The fragments of DNA (1.8 kb) were separated by electrophoresis using a 0.8% agarose gel made with TBE buffer. After separation the 1.8 kb 2C8 and 2C9 fragments of DNA were cut from the gel and electroeluted from the agarose gel into 1X TBE buffer. The TBE buffer containing the DNA was transferred to a 15 ml conical tube and the DNA precipitated overnight at -20°C by adding 1/10 volume 3M sodium acetate and an equal volume of isopropyl alcohol. The tube containing the DNA was centrifuged, the supernatant discarded, and the residue dissolved in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). The COS-1 expression vector pcDNA/AMP (Invitrogen, San Diego, CA) was digested with EcoRI to linearize the vector prior to ligation with the 2C8 or 2C9 cDNA. A 1.5 ml eppendorf tube containing 2 µl of pcDNA/AMP mini-prep DNA (about 2 µg), 3 µl React 3 buffer, 24 µl H2O, and 1 µl EcoRI was incubated at 37°C for 1 hour. To dephosphorylate the vector, 5 µl of 0.5M Tris pH 8.0, 14 µl of H2O, and 1 µl of bacterial alkaline phosphatase (BAP) (Gibco-BRL) was added to the reaction tube and incubated at 37°C for 30 minutes. After the incubation, BAP was removed by addition of an equal volume of buffer saturated phenol and 25 µl of chloroform:isoamyl alcohol (24:1), vortexing, centrifuging and transferring the organic phase containing the DNA to a clean tube. The DNA was precipitated by adding two volumes of ethanol. To ligate the 2C8 cDNA to the pcDNA/AMP vector,
2 μl of plasmid DNA, 4 μl of CYP2C8 mini-prep DNA, 1 μl of T4 DNA ligase (Gibco-BRL), 4 μl of 5X ligase buffer, and 9 μl of H₂O were added to a 0.5 ml eppendorf tube and incubated for 3 hours at 15°C. For ligation of 2C9 cDNA with pcDNA/AMP, 2 μl of plasmid DNA, 2 μl of CYP2C9 DNA, 1 μl of T4 DNA ligase (Gibco-BRL, 1 U/μl), 4 μl of 5X ligase buffer, and 11 of μl H₂O were added to a 0.5 ml eppendorf tube and incubated for 3 hours at 15°C.

The newly constructed 2C8- and 2C9-pcDNA/AMP vectors were used to transform E. coli.

Preparation of competent TOP10F’ E. coli:
To prepare competent TOP10F’ E. coli (Invitrogen), a 10 ml portion of LB broth was inoculated with a colony of the bacteria and the culture grown overnight at 37°C in a shaking water bath. A 100 μl aliquot of the bacterial culture was added to 10 ml of LB broth in a sterile 50 ml polypropylene tube and the culture incubated for another 2 hours at 37°C in a shaking water bath. The culture was placed on ice for 20 minutes, centrifuged at 4°C for 15 minutes at 4500 x g, the supernatant discarded and the cells resuspended in 5 ml of ice cold 50 mM CaCl₂ in 10 mM Tris-Cl, pH 8.0, with gentle swirling. The resuspended cells were placed on ice for 30 minutes, centrifuged at 4°C for 15 minutes at 4500 x g, the supernatant discarded and the cell pellet resuspended in 1 ml of ice cold 50 mM CaCl₂ in 10 mM Tris-Cl,
pH 8.0, with gentle swirling. These bacteria were used within 4 hours of preparation.

Transformation of TOP10F' *E. coli*:

For transformations of TOP10F' *E. coli*, 100 µl of the competent cells were pipetted into prechilled 1.5 ml eppendorf tubes along with 1 µg of purified plasmid DNA or 5 µl of a ligation reaction. The tubes were placed on ice for 30 min and heat shocked by incubating at 42°C for 2 min. One ml of LB broth was added to the tubes, and the cells were incubated at 37°C for 1 hr in a shaking water bath. Subsequently, 50-300 µl of the transformed *E. coli* were pipetted onto an LB agar plate containing 50 µg/ml ampicillin and the cells distributed over the plate with a glass spreader. The plates were incubated overnight at 37°C.

Isolation of pcDNA/AMP plasmid DNA from transformed TOP10F' *E. coli* by the mini-prep procedure:

Five ml cultures of LB broth containing 50 µg/ml ampicillin were inoculated with a colony of transformed *E. coli* and incubated overnight at 37°C. The cultures were poured into 1.5 ml eppendorf tubes and centrifuged for 10 min at 14,000 x g to pellet the bacteria. The supernatants were removed with Pasteur pipets, discarded, and the tubes inverted to drain. The sides of the tubes were wiped with a Kim-wipe to dry them completely. The pellets were
resuspended with 300 μl of STET (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), 5% Triton X-100) containing 1.5 mg lysozyme/ml by pipetting and placed on ice for 2 min. The tubes were heated in a boiling water bath for 1 min and immediately centrifuged at 14,000 x g for 10 min. The sticky pellets of chromosomal DNA and protein were removed from the bottom of the tube with wooden applicator sticks and discarded. The plasmid DNA remaining in the tubes was precipitated by adding 300 μl of 100% isopropanol, vortexed briefly, and placed in the -70°C freezer for 15-30 min. Following precipitation, the plasmid DNA was centrifuged for 10 min at 14,000 x g, the supernatants discarded and the pellets dried completely in a centrifugal evaporator with medium heat for 5 min. The DNA was resuspended in 50 μl TE or water.

Characterization of 2C8-pcDNA/AMP and 2C9-pcDNA/AMP by restriction analysis:

The plasmid DNA was digested with EcoRI to confirm that the 2C8 or 2C9 cDNA was ligated into the vector. Reactions contained 26 μl of mini-prep DNA, 3 μl of React 3 buffer and 1 μl of EcoRI. The tubes were incubated at 37°C for 4 minutes and 1 hour for 2C8 and 2C9 constructs, respectively. The plasmid DNA was digested with BamHI to determine the orientation of the 2C8 or 2C9 cDNA in the expression vector relative to the CMV promoter. For each digest, 26 μl of mini-prep DNA was incubated with 3 μl of React 3
buffer and 1 μl of BamHI for 1 hour at 37°C. The mRNA was degraded by adding 1 μl of 10 mg/ml RNaseA. The reactions were stopped by adding 1 μl of 0.5M EDTA, and the DNA was separated by electrophoresis on a 0.8% agarose gel. The size of the fragments from the restriction digest was compared to DNA fragments formed by the digestion of λ phage DNA with HindIII. The colonies of E. coli which contained plasmid DNA in the proper orientation were used to grow 500 ml cultures for isolation and purification of plasmid DNA. DNA was isolated and purified by the alkaline lysis and cesium chloride purification procedure. The 2C9-pcDNA/AMP expression plasmid is shown in Figure 3.

Isolation of plasmid DNA by the alkaline lysis/cesium chloride method:

A colony of transformed TOP10F' E. coli, picked from an agar plate, was used to seed a 5 ml culture of LB broth containing 50 μg/ml ampicillin. The broth culture was grown at 37°C for 1-2 hr, then used to seed a 500 ml culture of LB broth which was grown overnight at 37°C with shaking at 250 rpm. The 500 ml culture was transferred into two 250 ml bottles and the cells pelleted by centrifuging for 30 min at 4°C at 4500 x g. The supernatant was poured off and the pellet resuspended in 10 ml of 50 mM glucose/25 mM Tris (pH 8.0)/10 mM EDTA. A 20 ml aliquot of 0.2 M NaOH/1% SDS was added to each bottle and the cells were swirled to mix and left for 10 min at room temperature. A 15 ml portion of 3 M potassium acetate was
added to each bottle which was swirled to mix, incubated at room temperature for 5 min, and centrifuged for 20 min at 4°C at 4500 x g. The supernatant was filtered through two layers of gauze. The filtrate from both bottles was combined into a clean 250 ml bottle, and 0.6 volumes of isopropanol added. The bottle was inverted to mix and left overnight at -20°C to precipitate the DNA. The bottle containing the precipitated DNA was centrifuged at 4500 x g at 4°C for 20 minutes, the supernatant poured off, and the pellet resuspended in 8 ml of TE buffer with a pipet. The resuspended DNA was transferred to a 50 ml polypropylene centrifuge tube and 8.8 g of cesium chloride (Boehringer Mannheim, Indpls., IN) dissolved by gently inverting the tube. Upon solution of the cesium chloride, 0.8 ml of 10 mg/ml ethidium bromide was added and the tube inverted to mix. The DNA was transferred to polyallomer ultracentrifuge tubes and centrifuged in a Beckman L2B ultracentrifuge with a Beckman SW-60 rotor for 36 hr at 45,000 x g. The band of plasmid DNA was drawn from the centrifuge tube into a 3 ml syringe with an 18 gauge needle. The ethidium bromide was extracted from the DNA by vortexing with an equal volume of isopropanol equilibrated with 20X SSC (3 M NaCl, 0.3 M trisodium citrate), and washed with isopropanol until the wash appeared colorless. To remove the cesium chloride, the DNA was dialyzed against three 1-liter changes of TE buffer, allowing three hours for equilibration for each liter.
Figure 3. Plasmid for expressing CYP2C9 in COS-1 cells: The cDNA encoding CYP2C9 was cloned into the EcoRI site of the expression plasmid pcDNA/AMP. The expression of cytochrome P450 2C9 is regulated by the CMV promoter.
Maintenance of COS-1 cells

The media, Dulbecco’s modified Eagle media (DMEM) containing 4.5 g/L D-glucose, with L-glutamate, without sodium pyruvate, with 100 ml of heat inactivated fetal calf serum, 50 ml of 7.5% sodium bicarbonate, and 10 ml of 10,000 U/ml penicillin-streptomycin per liter, was replaced when the COS-1 cells (ATCC, Rockville, MD) reached confluence, typically 3-4 days. To split the cells, the media was removed from the plates and 1 ml or 3 ml of 0.05% trypsin, 0.53 mM EDTA were added to each 60 mm² or 100 mm² culture plate, respectively. After 2-3 minutes at room temperature, the solution of trypsin was removed and the plates placed in an incubator at 37°C with 5% CO₂ for 10-15 minutes. Four ml of media was added to each plate and the cells dissociated by pipetting. A 0.5 ml portion of the suspended cells was transferred to each new plate and 3 or 9 ml of fresh media added to the 60 mm² or 100 mm² plates, respectively and incubated at 37°C.

When cells were frozen for storage, the media was first removed from a plate of confluent cells and the cells dissociated by treating them with trypsin as described above. To resuspend the cells, 1.5 ml of filter-sterilized 90% fetal calf serum/10% DMSO was added to the plate and the cells resuspended by pipetting. The cells were transferred to three cryogenic tubes and frozen by placing on ice for 15 min, on dry ice for 15 min and finally stored at -70°C.
To start a culture from a tube of frozen cells, the tube was quickly thawed at 37°C and the cells transferred to a 60 mm² plate. Four ml of fresh media were added to the cells, which were maintained at 37°C in an incubator. In order to remove the DMSO from the cells, the media (4 ml) was replaced the following day.

Transfection of COS-1 cells:
COS-1 cells, grown in 60 mm² plates, were split 1:8 the day prior to transfection. Cells were transfected when they were 40 to 60% confluent, approximately 24 hours after splitting. The cells were transfected using 5 μg of DNA and 30 μl of Lipofectin per plate. The DNA and the Lipofectin were both diluted to 100 μl per plate with Opti-MEM. The diluted DNA and the Lipofectin were combined in a 15 ml polystyrene tube and incubated for 10-15 minutes at room temperature. To remove residual serum, the cells were washed twice with 1 ml of Opti-MEM. The DNA-Lipofectin mixture was diluted with 0.8 ml of Opti-MEM per plate and 1 ml of the mixture transferred to each 60 mm² plate of COS-1 cells. The cells were incubated at 37°C and after 16 hours the media replaced. After an additional 6 hours, DPH (1.25 or 1.5 μCi, representing 26.5 and 31.8 nmoles of DPH, respectively) was added to the 1 ml of media and the cells maintained in a 37°C for 30 to 48 hours. For control plates, the cells were transfected with Lipofectin diluted in Opti-MEM.
DPH metabolism in COS-1 cells:
The COS-1 cells, scraped with a rubber policeman from the plates, together with the 1 ml of media, were quantitatively pipetted into a 15 ml polypropylene centrifuge tube. The plates were quickly washed with 1 ml of acetonitrile (ACN) which was added to the cells and media in the 15 ml polypropylene tube. To precipitate the proteins, an additional 2 ml of ACN was added, the tube vortexed, centrifuged at 1800 x g for 10 minutes, and the supernatant transferred to 1.5 ml eppendorf tubes and dried in a centrifugal evaporator. A 1 ml portion of ACN was added to the 15 ml tubes containing the pellet and the tubes, vortexed, and centrifuged at 1800 x g for 10 minutes to extract additional DPH from the pellet, the supernatant was transferred to the tube containing the original supernatant, and dried in the centrifugal evaporator. This was repeated until the extractable radioactivity was less than twice background (approximately 90 dpm), typically 3-5 washes. The dried residue was reconstituted in 300 μl of mobile phase and analyzed by HPLC.

Construction of the 2C9-pRS315-pG1 yeast expression vector:
The 2C9-pRS315-pG1 vector was constructed by excising the 2C9 cDNA from the COS cell expression vector 2C9-pcDNA/AMP with EcoRI and blunt-end cloning into pRS315-pG1. To remove the 2C9 cDNA from pcDNA/AMP, 3.8 μl of CsCl purified 2C9-pcDNA/AMP (approximately 5 μg), 2 μl of 10X
NEBuffer 3, 2 µl of 10 mg/ml acetylated BSA, 2 µl of EcoRI (New England Biolabs (NEB), Beverly, MA, 20 U/µl), and 10.2 µl of H₂O were added to a 1.5 ml eppendorf tube and heated at 37°C for 1 hour. The ends of the fragment encoding 2C9 were made blunt by adding 2 µl of 330 µM dNTPs (dATP, dCTP, dGTP, dTTP), 0.5 µl of the large fragment of T4 DNA polymerase (Klenow fragment, NEB, 3 U/µl) and incubating the mixture at room temperature for 15 minutes. The enzymes were inactivated by heating at 65°C for 10 minutes. The pRS315-pG1 vector, from Dr. T.H. Chang (Dept. of Molecular Genetics, Ohio State University, Columbus, OH) contains a unique BamHI restriction site between the promoter and terminator. The vector was linearized by adding 3 µl of plasmid DNA, 2 µl of 10X NEBuffer 3, 2 µl of 10 mg/ml acetylated BSA, 2 µl of BamHI, and 14 µl of H₂O and incubating at 37°C for 1 hour. The ends of the vector were made blunt by adding 2 µl of 330 µM dNTPs and 0.5 µl of Klenow fragment and incubating at room temperature for 15 minutes. The enzymes were inactivated by heating at 65°C for 10 minutes. The blunt-end vector was dephosphorylated to avoid self-ligation by adding 1 µl of calf intestinal alkaline phosphatase (CIAP, NEB, 10 U/µl) and heating at 37°C for 1 hour. The digested 2C9-pcDNA/AMP and pRS315-pG1 were separated on a 0.8% agarose gel made with TAE buffer. The size of the digested DNA fragments was determined by comparison to 1 µg of λ/HindIII DNA standards (Gibco-BRL) made in NEBuffer 3. The desired fragments of DNA (1.8 and 6.8 kB) were purified from the gel
by elution onto DEAE membranes (General Method 1). The 2C9 fragment and the pRS315-pG1 fragment were ligated by adding 2 μl of vector DNA, 4 μl of 2C9 DNA, 2 μl of H2O, 1 μl of 5X T4 DNA ligase buffer (NEB), and 1 μl of T4 DNA ligase (NEB, 20 U/μl). Duplicate ligation reactions contained 2 μl of vector DNA, 6 μl of 2C9 DNA, 1 μl of 5X T4 DNA ligase buffer, and 1 μl of T4 DNA ligase in a reaction volume of 10 μl. These were incubated at 16°C for 16 hours and used to transform XL-1 Blue supercompetent *E. coli* (Stratagene, La Jolla, CA) (General Method 3). Several colonies of *E. coli* were picked from the LB/AMP plates and used to start LB/AMP broth cultures for isolation of plasmid DNA by the mini-prep procedure (General Method 4). The mini-prep DNA was used to characterize the plasmid DNA by restriction digestion, the polymerase chain reaction (PCR) and sequencing.

**Construction of the 2C9-pRS426-pG1 yeast expression vector:**

To construct the 2C9-pRS426-pG1 vector, 2C9-pRS315-pG1 was cut with the restriction enzymes NotI and XhoI to remove the 3.2 kb GPD promoter-2C9 insert-PGK terminator portion of the plasmid. This NotI-XhoI fragment was ligated into the pRS426-pG1 vector, which had been linearized with NotI and XhoI. The pRS426-pG1, from Dr. T-H. Chang, contains a unique BamHI site between the promoter and the terminator. To remove the 3.2 kb fragment from the pRS315-pG1, 3 μl of mini-prep DNA was pipetted into a 1.5 ml eppendorf tube with 2 μl of 10X NEBuffer 2, 2 μl of 1 mg/ml
acetylated BSA, 11.8 μl of H₂O, 0.2 μl of RNase I, 0.5 μl Notl (NEB, 10 U/μl) and 0.5 μl Xhol (NEB, 20 U/μl). The tube was incubated at 37°C for 1 hour and the reaction stopped by heat-inactivation for 10 minutes at 65°C. Following the addition of 4 μl of loading buffer, the DNA was separated on a 0.8% agarose gel. The region containing the 3.2 kB fragment was cut from the gel to remove this fragment and purified by the Geneclean method (General Method 2). The vector was digested with Notl and Xhol to remove the 1.4 kb promoter and terminator portion of the plasmid. To remove the fragment, 3 μl of mini-prep DNA was pipetted into a 1.5 ml eppendorf tube with 2 μl of NEBuffer 2, 2 μl of 1 mg/ml acetylated BSA, 0.2 μl of RNase, 11.8 μl of H₂O, 0.5 μl of Notl and 0.5 μl of Xhol. The reactions were incubated at 37°C for 1 hour, heat-inactivated for 10 minutes at 65°C and the vector DNA dephosphorylated by adding 1 μl of calf intestinal alkaline phosphatase (CIAP) and heating at 37°C for 1 hour. An additional 1 μl of CIAP was added after 30 minutes. The digested and dephosphorylated DNA was separated on a 0.8% agarose gel after the addition of 4 μl of loading buffer. The section of the gel containing the linearized vector (7 kB) was cut out and the DNA isolated by the Geneclean method (General Method 2). The purified vector and the 3.2 kb DNA fragment containing the promoter, 2C9 cDNA, and terminator were ligated to form the 2C9-pRS426-pG1 plasmid. To ligate the fragments, two reaction conditions were used: 2 μl of vector DNA was added to 500 μl thin-walled tubes with 4 or 6 μl of insert DNA, 5
or 3 μl of H₂O, 3 μl of 5X ligase buffer (Gibco-BRL), and 1 μl of T4 DNA ligase (Gibco-BRL, 1 U/μl) for a total volume of 15 μl. A control reaction contained 2 μl of vector DNA, 3 μl of 5X ligase buffer, 9 μl of H₂O, and 1 μl of T4 DNA ligase. The reactions were incubated for 16 hours at 16°C in a thermal cycler. A 5 μl portion of the ligation reactions was used to transform supercompetent XL-1 Blue E. coli (General Method 3) and 100 μl of the transformed cells spread on LB/AMP plates. The plates were incubated overnight at 37°C. Several colonies of E. coli were picked from the LB/AMP plates and used to inoculate LB broth cultures for the isolation of plasmid DNA by the mini-prep procedure (General Method 4). This mini-prep DNA was used to characterize the plasmid by restriction analysis, PCR and sequencing. The mini-prep, plasmid DNA which had the appropriate construction was used to transform S. cerevisiae strain BJ5460 (General Method 5). The 2C9-pRS426-pG1 expression plasmid is shown in Figure 4.

Characterization of 2C9-pRS315-pG1 and 2C9-pRS426-pG1 by restriction analysis:

Colonies of XL-1 Blue E. coli, transformed with 2C9-pRS315-pG1 or 2C9-pRS426-pG1 liggations, were picked from the LB/AMP agar plates and used to seed 2 ml LB/AMP broth cultures for isolation of plasmid DNA by the boiling mini-prep method (General Method 4). The 2C9-pRS315-pG1 and 2C9-pRS426-pG1 mini-prep DNA were digested with the restriction enzymes Nael
Figure 4. Plasmid 2C9-pRS426-pG1 for expressing CYP2C9 in S. cerevisiae: The cDNA encoding CYP2C9 was cloned into a BamHI restriction site between the constitutive GPD promoter and the PGK terminator of the expression vector pRS426-pG1. The plasmid contains the URA3 nutritional selection marker, the 2μm origin of replication and the ampicillin resistance gene.
and Pvull to confirm the presence and orientation of the 2C9 cDNA in the vectors relative to the GPD promoter. The orientation of the 2C9 cDNA relative to the promoter should be unchanged by cloning into the pRS426-pG1 vector. Restriction digests with Nael contained 3 μl of mini-prep DNA, 2 μl of 10X NEBuffer1, 2 μl of 10 mg/ml BSA, 0.2 μl of RNaseI, 12.3 μl of H2O, and 0.5 μl of Nael. Restriction digests with Pvull contained 3 μl of mini-prep DNA, 2 μl of 10X NEBuffer1, 2 μl of 10 mg/ml BSA, 0.2 μl of RNaseI, 12.3 μl of H2O, and 0.5 μl of Pvull. The reactions were incubated at 37 °C for 1 hour, and separated on a 0.8% agarose gel. The DNA fragments from the restriction digests were compared to 1 μg of a 1 kb DNA ladder (Gibco-BRL) diluted in NEBuffer 1.

Characterization of 2C9-pRS315-pG1 and 2C9-pRS426-pG1 by PCR:
The polymerase chain reaction was used to amplify the DNA between a primer complementary to the promoter region of the plasmid and a primer complementary to the 5' end of the 2C9 cDNA to determine the orientation of the 2C9 cDNA relative to the GPD promoter. The appearance of a DNA fragment would indicate that the cDNA was inserted into the vector in the correct orientation. The T7 primer had the sequence 5' d[TAA TAC GAC TCA CTA TAG GG] 3' and was complementary to the terminator region of the pRS315-pG1 and pRS426-pG1 plasmids. The reverse primer had the sequence 5' d[CAG GAA ACA GCT ATG AC] 3' and was complementary to
the promoter regions of the pRS315-pG1 and pRS426-pG1 plasmids. The P450-1 primer, complementary to the 5’ end of the cDNA encoding 2C9, had the sequence 5’ d[GCT CTG TCT CCA GAG TGA] 3’. The primers were purchased from Amitof Biotech (Boston, MA). The total volume of each reaction was 100 \( \mu l \). Each reaction tube contained 1 \( \mu l \) of template mini-prep DNA, diluted 1:1000 with \( \text{H}_2\text{O} \), 10 \( \mu l \) of 10 \( \mu \text{M} \) upstream and downstream primers, 10 \( \mu l \) of 10X Taq polymerase buffer (Perkin Elmer, Branchburg, NJ), and 5 \( \mu l \) of 50 mM magnesium chloride. The tubes were heated at 92°C for 10 minutes, chilled on ice, and 10 \( \mu l \) of a 2 mM stock solution of deoxynucleotides and 1 \( \mu l \) of Ampli-Taq (Perkin-Elmer) added to each tube. For the first cycle the tubes were heated at 92°C for 10 minutes. For 30 cycles the tubes were heated at 92°C for 1 minute, 45°C for 30 seconds, and 72°C for 1 minute. Following the last temperature cycle, the tubes were heated at 72°C for 10 minutes, then chilled to 4°C. To remove the mineral oil, 150 \( \mu l \) of chloroform was added, the tubes vortexed, centrifuged, and the upper aqueous phase transferred to a clean tube. To visualize the PCR products, 10 \( \mu l \) of the amplified DNA was combined with 2 \( \mu l \) of 10X React3 buffer and 8 \( \mu l \) of water and the samples separated on a 2% agarose gel.

Sequencing 2C9-pRS315-pG1 and 2C9-pRS426-pG1 double-stranded DNA
The products of sequencing reactions were separated on a 6% polyacrylamide gel. The glass plates for the sequencing gel apparatus (BRL
Model S2) were washed with soap and water, siliconized with 20 ml of dimethyl-dichlorosilane in chloroform per plate, and placed in the hood for several minutes to evaporate the chloroform. Spacers and sponges between the plates were fastened with gel tape. The plates were elevated slightly to permit the gel to be poured. To make the gel, 60 ml of stock acrylamide, 10 ml of buffer, and 600 μl of 10% ammonium persulfate were added to a flask, mixed gently, and poured between the glass plates with a 60 ml syringe. The reagents for the gel were from a Sequagel-6 kit (National Diagnostics, Atlanta, GA). The comb was placed in the gel, and the gel left at room temperature overnight to polymerize.

To sequence double-stranded DNA, 1 μl of 10 μM primer complementary to the template DNA was combined with 10.5 μl of mini-prep DNA in a 1.5 ml eppendorf tube. The tube was boiled for 3 minutes in a water bath, placed on ice for 5 minutes, and centrifuged for 1 minute to precipitate the condensation from the sides of the tubes. The amount of each reagent added to the reactions was determined by the number of DNA templates to be sequenced. The amount of each reagent was calculated by using a formula where X was equal to 1/2 the number of templates to be sequenced. For example, for sequencing 10 templates, X = 5. To a 1.5 ml eppendorf tube, X μl of [35S]dATP, 2X μl of 0.1M dithiothreitol, 4X μl of 5X sequencing buffer, 0.8X μl of dGTP labelling mix, and 0.5X μl of Sequenase were added. The
sequencing buffer, dGTP labelling mix and Sequenase were purchased as a kit from United States Biochemical (Cleveland, OH). The $[^{35}\text{S}]$dATP was purchased from New England Nuclear (Boston, MA). The tube was mixed gently, 4.1 µl of the reaction mix added to the tube containing the annealed DNA and primer, and the tube incubated at room temperature for 5 minutes to allow elongation of the primer on the DNA template. A 3.5 µl portion of the elongation reaction was added to 2.5 µl of ddG, ddA, ddT and ddC termination mix, in separate eppendorf tubes, and the tubes incubated at 37°C for 5 minutes. The reactions were stopped by adding 4 µl of stop buffer. The samples were heated at 75°C for 2 minutes and chilled on ice. Three µl of the reactions were loaded on the 6% sequencing gel which had been prerun at 60W for 10-15 minutes. The gel was run with TBE buffer at 60W with constant voltage until the upper xylene cyanol band of the loading buffer had traveled 3/4 of the length of the gel. When the electrophoresis was complete, the upper buffer compartment was drained and the glass plates removed from the apparatus. The top glass plate was gently pried off the gel and a piece of Whatman 3MM filter paper placed on the gel. The gel was turned over with the filter paper on the bottom and the second glass plate removed. The gel was covered with Saran wrap and dried for 30 minutes under vacuum on a gel dryer. The dried gel was aligned with a piece of XAR-2 X-ray film (Eastman Kodak Co., Rochester, NY) in a light-tight exposure cassette. The film was developed after an overnight exposure to
the gel and the sequence read manually on a light box.

**Construction of the 2C18-pRS426-pG1 yeast expression plasmid**

The cDNA for the expression of cytochrome P450 2C18 was a gift from Dr. F. Gonzalez (Laboratory of Molecular Carcinogenesis, NCI, NIH) (91). To construct the 2C18-pRS426-pG1 vector, the 1.75 kB fragment containing the CYP2C18 cDNA was cut out of the 2C18-pUC19 vector with the restriction enzymes Asel and Narl. This Asel-Narl fragment was blunt-end ligated into the pRS426-pG1 vector, which was linearized with BamHI. To remove the 1.75 kB fragment from pUC19, 5 µl of mini-prep DNA was pipetted into a 1.5 ml eppendorf tube with 2 µl of 10X NEBuffer 1, 2 µl of 1 mg/ml acetylated BSA, 0.5 µl of RNase I, 9.8 µl of H₂O, and 0.5 µl of Narl (NEB, 2-5 U/µl). The tube was incubated at 37 °C for 1.5 hours and heat-inactivated at 65 °C for 10 minutes. To change the buffer for the Asel digest, 2 µl of 1M sodium chloride was added to the reaction tube together with 1 µl of Asel (NEB, 10 U/µl). The tube was incubated at 37 °C for 1 hour and the reaction stopped by heat-inactivating at 65 °C for 10 minutes. The ends of the fragment were made blunt by adding 2 µl of 2 mM dNTPs and 0.5 µl of Klenow fragment to the tubes and incubating for 15 minutes at room temperature. The incubations were combined with 4 µl of loading buffer and separated on a 0.8% agarose gel. The region containing the 1.75 kB fragment was cut from the gel and purified by the Geneclean method (General Method 2).
pRS426-pG1 miniprep DNA was digested with BamHI to linearize the vector (7 kB) by adding 5 µl of mini-prep DNA to a 1.5 ml eppendorf tube with 2 µl of 10X React 3 buffer, 2 µl of 1 mg/ml acetylated BSA, 0.2 µl of RNase I, 10.3 µl of H₂O and 0.5 µl of BamHI (Gibco-BRL, 10 U/µl). The reactions were incubated at 37 °C for 1 hour and heat-inactivated for 10 minutes at 65 °C. The ends of the vector were blunted by adding 2 µl of 2 mM dNTPs and 0.5 µl of Klenow fragment to the tubes and incubating for 15 minutes at room temperature. The vector was dephosphorylated by adding 1 µl of CIAP to the tubes and incubating at 37 °C for 30 minutes. Another 1 µl of CIAP was added to the tubes, which were incubated for 30 minutes at 37 °C. Following the addition of 4 µl of loading buffer, the DNA was separated on a 0.8% agarose gel. The portion of the gel containing the linearized vector was cut out and the DNA isolated by the Geneclean method (General Method 2). The purified vector (7 kB) and the 1.75 kB DNA fragment containing the CYP2C18 cDNA were ligated to form the 2C18-pRS426-pG1 plasmid. To ligate the fragments, two reaction conditions were used: 2 µl of vector DNA was added to 500 µl thin-walled tubes with 6 µl of 2C18 DNA, 3 µl of 5X ligase buffer, 3 µl of H₂O, and 1 µl of T4 DNA ligase, or 2 µl of vector DNA, 10 µl of 2C18 DNA, 4 µl of 5X ligase buffer, 3 µl of H₂O, and 1 µl of T4 DNA ligase. The ligation reactions were incubated for 16 hours at 16 °C in a thermal cycler. A 5 µl portion of each ligation reaction was used to transform supercompetent XL-1 Blue E. coli (General Method 3). The plates of
transformed cells were incubated overnight at 37°C. Several colonies were picked from the LB/AMP plates and used to inoculate LB broth cultures for the isolation of plasmid DNA by the mini-prep procedure (General Method 4). This mini-prep DNA was used to characterize the plasmid by restriction analysis. The mini-prep DNA which had the correct restriction fragments was used to transform *S. cerevisiae* strain BJ5460 for metabolism studies of DPH (General Method 5).

**Construction of the 2C8-pRS426-pG1 yeast expression plasmid:**

The cDNA encoding cytochrome P450 2C8 was cut out of the expression vector 2C8-pcDNA/AMP with the restriction enzymes HindIII and Xhol and blunt-end ligated into the pRS426-pG1 vector, linearized with BamHI. To remove the 1.8 kB fragment from pcDNA/AMP, 5 µl of mini-prep DNA was pipetted into a 1.5 ml eppendorf tube with 2 µl of 10X React3 buffer, 2 µl of 1 mg/ml acetylated BSA, 0.5 µl of RNase I, 8.5 µl of H₂O, 1 µl of HindIII, and 1 µl of Xhol. The tube was incubated at 37°C for 1 hour, the enzymes heat-inactivated at 65°C for 10 min, and the DNA fragment blunted by addition of 2 µl of 2 mM dNTPs and 0.5 µl of Klenow fragment to the tubes and incubation for 15 min at room temperature. The pRS426-pG1 vector DNA (7 kB) was linearized by adding 5 µl of mini-prep DNA to a 1.5 ml eppendorf tube with 2 µl of 10X React3 buffer, 2 µl of 1 mg/ml acetylated BSA, 0.5 µl of RNase I, 9.5 µl of H₂O, and 1 µl of BamHI and incubating at 37°C for 1
hour. The enzymes were heat-inactivated at 65°C for 10 min, and the ends blunted by addition of 2 µl of 2 mM dNTPs and 0.5 µl of Klenow fragment and incubation at room temperature for 15 minutes. The vector was dephosphorylated by adding 1 µl of CIAP to the tubes and incubating at 37°C for 30 min. Another 1 µl of CIAP was added to the tubes, and they were incubated an additional 30 min. The vector (7 kB) and DNA encoding 2C8 (1.8 kB) were separated by electrophoresis using a 0.8% agarose gel. The fragments were isolated and purified by the DEAE membrane method (General Method 1). The purified fragment of DNA encoding cytochrome P450 2C8 and the vector were ligated by combining 6 µl of purified 2C8 DNA, 2 µl of vector DNA, 1 µl of T4 DNA ligase, 3 µl of T4 DNA ligase buffer, and 3 µl of H₂O in 500 µl thin-walled tubes and incubating overnight at 16°C in a thermal cycler. The orientation of the 2C8 cDNA in the vector was determined by restriction analysis of mini-prep DNA with Ncol and Pvull.

Construction of the b₅-pRS426-pG1 yeast expression plasmid:
The expression plasmid b₅-pRS426-pG1 was constructed by removing the 405 bp fragment of DNA encoding cytochrome b₅ from the plasmid pUC19 with BglII and EcoRI and blunt-end cloning into the BamHI site of the expression plasmid pRS426-pG1. The cDNA encoding cytochrome b₅ was obtained from Drs. P. Urban and D. Pompon (Centre de Genetique Moleculaire, Gif-sur-Yvette, France). The DNA encoding cytochrome b₅ was
removed by adding 5 μl of mini-prep DNA to a 1.5 ml eppendorf tube with 2 μl of 10X React3 buffer, 2 μl of 1 mg/ml acetylated BSA, 0.5 μl of RNase I, 8.5 μl of H₂O, 1 μl of BglII, and 1 μl of EcoRI and heating at 37°C for 1 hour. The enzymes were heat-inactivated for 10 minutes at 65°C, and the ends of the DNA blunted by addition of 2 μl of 2 mM dNTPs and 0.5 μl of Klenow fragment and incubation at room temperature for 30 minutes. The pRS426-pG1 vector (7 kB) was linearized by adding 5 μl of mini-prep DNA to a 1.5 ml eppendorf tube with 2 μl of 10X React3 buffer, 2 μl of 1 mg/ml acetylated BSA, 0.5 μl of RNase I, 9.5 μl of H₂O, and 1 μl of BamHI and incubating at 37°C for 1 hour. The enzymes were heat-inactivated at 65°C for 10 minutes, and the ends of the DNA blunted by addition of 2 μl of 2 mM dNTPs and 0.5 μl Klenow fragment and incubation at room temperature for 30 minutes. The vector was dephosphorylated by adding 1 μl CIAP and incubating for 30 minutes, followed by another 1 μl of CIAP and incubation for an additional 30 minutes. Four μl of loading buffer were added to each tube and the DNA fragments separated by electrophoresis on a 0.8% agarose gel. The DNA fragments (405 bp and 7 kB) were purified by the DEAE membrane technique (General Method 1). The linearized vector and the DNA fragment encoding cytochrome b₆ were ligated by adding 3 μl of purified DNA encoding cytochrome b₆, 2 μl of vector DNA, 1 μl of T4 DNA ligase, 2 μl of T4 DNA ligase buffer, and 2 μl of H₂O to thin-walled 500 μl tubes and incubating overnight at 16°C in a thermal cycler. The orientation of the
cytochrome b₆ cDNA in the vector was determined by restriction analysis of mini-prep DNA with BstXI and PstI.

General Methods applied for expression of cytochrome P450 enzymes in *S. cerevisiae*

1. Purification of DNA with DEAE membranes:

Prior to use, DEAE membranes (Schleicher and Schuell, Keene, NH) were prepared by incubating at room temperature for 10 minutes in 200 µl of 10 mM EDTA (pH 7.6), for 5 minutes in 200 µl of 0.5 M NaOH, and washed 3 times with 200 µl distilled water. The DNA to purify was separated on a 0.8% agarose gel to resolve the bands of DNA. The gel was loaded with at least one empty lane between samples. The gel was placed on a uv light box to visualize the location of the DNA and, with a clean razor, a slit cut in the gel perpendicular to the band. The prepared DEAE membrane was inserted into the slit in the agarose gel with forceps, the excess gel removed, the remaining gel placed into the electrophoresis chamber at a 90° angle to the original orientation (the membrane on the anode side of the band), and the DNA eluted onto the membrane. The DEAE membrane was removed from the gel with forceps, placed in a 1.5 ml centrifuge tube, and rinsed twice with 200 µl of 0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris (pH 8.0). The DNA was eluted from the membrane by addition of 200 µl of 1M NaCl, 0.1 mM EDTA, 20 mM Tris (pH 8.0) and incubation for 20 minutes at 55-65°C. The elution
of the DNA from the membrane was monitored by viewing with long wavelength uv light (365 nm). After the DNA was eluted, the membrane was rinsed with 100 µl of 1M NaCl, 0.1 mM EDTA, 20 mM Tris (pH 8.0). The two elution solutions were combined, centrifuged at 15,000 x g for 5 minutes, and the DNA precipitated by adding 750 µl of ethanol and 2 µl of 1 mg/ml glycogen (Boehringer Mannheim) and cooling overnight at -20°C. The tubes were centrifuged at 15,000 x g for 15 minutes to pellet the DNA. The pellets were dried for 5 minutes in a centrifugal evaporator and dissolved in 20 µl water.

2. Purification of DNA from agarose with Geneclean:

The Geneclean kit was purchased from Bio101 (La Jolla, CA). DNA fragments were separated on a 0.8% agarose gel, the gel placed on a uv light box and the bands to be isolated removed by cutting with a razor blade. The gel slice was transferred to a 1.5 ml centrifuge tube and centrifuged briefly to pellet the agarose. For gels made with TAE, three volumes of NaI were added to the gel slice and the slice heated at 50-65°C for 5 minutes to dissolve the agarose. For gels made with TBE, 0.5 volumes of TBE modifier and 4.5 volumes of NaI were added. To bind the DNA, 5-10 µl of glassmilk were added to each tube. The glassmilk was suspended every 1 to 2 minutes by pipetting and after 5 minutes, the tube centrifuged for 5 seconds to pellet the glassmilk. The supernatant containing the agarose and NaI was aspirated
and the pellet washed three times with 500 µl of NEW buffer. To wash the pellets, NEW buffer was added to each tube, the pellet resuspended by pipetting, the tube centrifuged for 5 seconds to pellet the glassmilk, and the NEW buffer aspirated. To elute the DNA, 15-20 µl of water was added and the pellet resuspended by pipetting. The tubes were heated at 55-65°C for 3 minutes, centrifuged for 30 seconds at 14,000 x g and the supernatant containing the DNA transferred to a clean tube. The glassmilk was washed with an additional 15-20 µl of water and the supernatants combined.

3. Transformation of *E. coli* with pRS426 vectors:

To transform supercompetent XL-1 Blue *E. coli* (Stratagene, La Jolla, CA), 50 µl of cells were thawed on ice and added together with 0.85 µl of fresh β-mercaptoethanol to prechilled 1.5 ml eppendorf tubes. The cells, kept on ice for 10 minutes, were mixed every 2 minutes by pipetting. Approximately 0.5 µg of purified plasmid DNA or 5 µl of a ligation reaction were added to the cells and incubated on ice for 30 minutes. The cells were heat shocked for 3 minutes at 42°C and placed on ice for 2 minutes. A 0.45 ml portion of prewarmed SOC media (9.7 ml LB broth, 20 mM MgCl₂, 2.5 mM KCl, 0.4% glycerol in 10 ml final volume) was added to each tube and the cells grown for 1 hr at 37°C. The cells were streaked on LB agar plates containing 50 µg/ml ampicillin. The volume of cells used to streak the plates varied from 50 µl for purified plasmids to 500 µl for blunt-end ligation reactions. Often when
a large number of cells were used to streak the plates, the cells were briefly
centrifuged at 5000 x g and a portion of the resulting supernatant removed
with a pipet and discarded. The cells were resuspended in the remaining
supernatant, transferred to the plates with a pipet and distributed over the
plate with a glass spreader. The plates were incubated overnight at 37 °C to
allow colonies to grow.

4. Isolation of pRS315 and pRS426 plasmid DNA from transformed E. coli
by the mini-prep procedure:
A 2 ml portion of LB broth containing 50 μg/ml ampicillin was transferred to
a sterile 15 ml polypropylene tube, inoculated with a colony of E. coli,
incubated overnight in an orbital shaking water bath at 37 °C at 250 rpm, and
a portion poured into a 1.5 ml eppendorf tube. The tube was centrifuged for
2 minutes to pellet the cells, the supernatant aspirated and discarded, and the
pellet resuspended in 300 μl of STET (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0),
1 mM EDTA (pH 8.0), 5% Triton X-100) by vortexing for 3 minutes. A 25
μl portion of 10 mg/ml lysozyme was added to the tube which was inverted
2-3 times, incubated at room temperature for 10 minutes, heated at 100 °C
in a heat block, and centrifuged immediately for 15 minutes at 14,000 x g.
The sticky pellet of chromosomal DNA and protein was removed from the
bottom of the tube with a wooden applicator stick and discarded. The
plasmid DNA remaining in the tube was precipitated by addition of 300 μl of
2.5 M ammonium acetate, 75% isopropanol, inverted, and placed at 4°C for 20 minutes. Following precipitation, the plasmid DNA was centrifuged for 15 minutes at 14,000 x g, the supernatant aspirated and discarded, the tube centrifuged for 2 minutes at 14,000 x g, and the supernatant removed with a pipet and discarded. The pellet was rinsed with 750 µl of 70% ethanol, the tube inverted 2-3 times, the supernatant poured off, the tube centrifuged at 14,000 x g for 2 minutes, and the supernatant removed with a pipet. The remaining ethanol was removed from the pellet in a centrifugal evaporator for 5 minutes, and the pellet dissolved in 50 µl H₂O.

5. Transformation of *S. cerevisiae* with plasmid DNA

The BJ5460 strain of *S. cerevisiae*, obtained from Dr. T-H. Chang (Dept. Molecular Genetics, Ohio State University, Columbus, OH) was grown in yeast peptone broth with dextrose (YPD). This broth contained 20 g peptone and 10 g yeast extract per liter and 2% w/v dextrose. To grow the *S. cerevisiae*, 40 ml of sterile YPD was transferred to a 250 ml Erlenmeyer flask and seeded with a colony of wild type yeast. The culture was grown overnight at 30°C in a shaking water bath to reach an OD₆₀₀ of 1-3. To measure the OD₆₀₀, a 100 µl portion of the culture was transferred to a culture tube and diluted with 900 µl of water. The absorbance of the diluted culture was measured against a water blank. The cells were transferred to a 50 ml culture tube, centrifuged at 4500 x g for 5 minutes, and the
supernatant removed by pouring. The pellet was resuspended by addition of 2 ml of freshly made TE/LiOAc (10 mM Tris, 1 mM EDTA, and 100 mM lithium acetate), added to make the cells porous. The cells were precipitated by centrifuging at 4500 x g for 5 minutes, washed with another 2 ml TE/LiOAc, and the cells resuspended in 0.8 ml of TE/LiOAc. The cells were used immediately or stored at 4°C for a week. To transform yeast, 100 μl of competent yeast were combined with 2.5 μl of mini-prep DNA and 5 μl of sheared salmon sperm DNA. The tubes were mixed by tapping and incubated at 30°C for 30 minutes. To each tube, 0.65 ml of PEG mix (3.2 ml of 50% PEG 4000, 0.4 ml 100 mM Tris/10 mM EDTA, and 0.4 ml 1 M lithium acetate) was added, mixed by pipetting, and the tubes incubated at 30°C for 1 hour. The cells were heat-shocked at 42°C for 15 minutes and centrifuged at 3000 x g for 1 minute to precipitate. The supernatant was removed and the cells resuspended in 200 μl of sterile water. The whole transformation mixture was spread on the appropriate 100 mm² agar selection plate. Transformed yeast were grown on SD + amino acid agar plates. SD media contained 6.7 g yeast nitrogen base without amino acids (Difco, Detroit, MI) and 2% w/v dextrose. A 10 X stock solution of amino acids was prepared with 200 mg uracil, 300 mg L-leucine, 200 mg L-histidine, 200 mg adenine sulfate, 300 mg L-lysine, 200 mg L-tryptophan, 200 mg L-methionine, 300 mg L-tyrosine per liter of water. The adenine sulfate was from ICN (Aurora, OH) and all other amino acids were from Sigma. The solution was stirred and
heated at 60°C to dissolve the amino acids. Amino acid stock solutions were prepared without L-leucine or uracil for growing *S. cerevisiae* transformed with pRS315-pG1 or pRS426-pG1, respectively. Agar plates were made by adding 25 g agar to the SD media, autoclaving and pouring into 100 mm² plates prior to cooling. The plates were incubated for 3 days at 30°C to allow the colonies to grow.

6. Growth of *S. cerevisiae*:

To grow transformed *S. cerevisiae* for making microsomes, a 15 ml aliquot of SD media containing the correct amino acids was transferred to a 50 ml polypropylene tube and seeded with a single colony of transformed yeast picked from the agar plate. The culture was grown at 30°C to saturation (OD₆₀₀ approximately 3.0) in a shaking water bath. The transformed cells took approximately 40 hours to reach saturation. Five ml of the saturated culture was used to seed 1 liter of SD broth containing the appropriate amino acids and the cells grown in Fernbach flasks to increase the aeration. The cultures were grown in a shaking water bath at 30°C until the absorbance at 600 nm reached 1.6-1.8. The transformed cells took approximately 16-20 hours to reach this density. The cells were transferred to four 500 ml polypropylene centrifuge bottles and centrifuged at 4500 x g for 15 min at 4°C to pellet the cells and the supernatant poured off. Cells that were not used immediately were frozen at -20°C as a pellet. Typically four 1-liter
cultures were grown for the preparation of microsomes.

7. Preparation of microsomes from S. cerevisiae:
The microsomes of yeast expressing the cytochrome P450 enzymes were prepared by modification of the method described by Goldstein (104). The buffers for preparation of microsomes of yeast were as follows: Buffer A consisted of 10 mM Tris, pH 7.5, 0.65 M sorbitol, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT). The DTT was added immediately before use. Buffer B consisted of 10 mM Tris, pH 7.5, 2 M sorbitol, 0.1 mM EDTA, 1 mg/ml zymolyase 20T or 0.2 mg/ml zymolyase 100T (ICN, Aurora, OH), and 0.1 mM DTT. The zymolyase, added to digest the cell wall, and the DTT, added to activate the zymolyase, were added immediately before use. Sonication buffer contained 100 mM potassium phosphate, pH 7.4 and 0.1 mM EDTA and resuspension buffer contained 100 mM potassium phosphate pH 7.4, 0.1 mM EDTA, and 20% glycerol.

The cells grown to mid-log phase and centrifuged were used to prepare microsomes. To each centrifuge bottle, 20 ml of Buffer A was added and the pellet resuspended by pipetting. The bottles were centrifuged at 4500 x g at 4°C to pellet the cells. The supernatant was poured off, 20 ml of Buffer B added to each bottle, and the bottles shaken at 30°C in an orbital shaker at 150 rpm for 1 hour to dissolve the cell walls. The spheroplasts, yeast cells
without their cell wall, were centrifuged at 4500 x g for 15 minutes at 4°C and the supernatant poured off. Twenty ml of sonication buffer were added to each bottle and the pellets resuspended by pipetting. The spheroplasts were transferred to a glass or metal beaker and sonicated with a tapered tip probe and a Tekmar 50 W sonicator, set at 40. The beaker was kept on ice during the sonication to avoid overheating. The spheroplasts were sonicated for eight 1-minute bursts with 1 minute intervals between each burst to allow the sample to cool. The cell lysate was transferred to 50 ml round-bottom Sorvall polypropylene centrifuge tubes and centrifuged at 9000 x g for 30 minutes in a Beckman Type 65 rotor. The supernatant, transferred to ultracentrifuge tubes, was centrifuged at 105,000 x g for 90 minutes. The supernatant was poured off and discarded and the pellets resuspended in 500 µl resuspension buffer per liter of initial culture. The concentration of protein in 1:25 and 1:50 dilutions of the microsomes was determined by the BCA method. Bovine serum albumin (Sigma) dissolved in resuspension buffer was used as protein standards. The microsomes were stored at -70°C until used for incubations.

8. BCA protein assay

The concentration of proteins in microsomes was determined by the Pierce BCA assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard in the range of 0 to 1000 µg/ml. The absorbance of the samples and
standards was determined in a Bausch and Lomb spectrophotometer at 562 nm using water as a reference. The absorbance of the standards was plotted against the concentrations and the best fit line (polynomial line $y = ax + bx^2 + cx^3$) was calculated with the Inplot program (GraphPad, San Diego, CA). Correlation coefficients for the standard curves were greater than or equal to 0.999.

9. SDS-PAGE electrophoresis of proteins

Proteins from microsomes were separated by SDS-PAGE based on the method of Laemmli (133). To separate proteins, 10% SDS-polyacrylamide gels were poured or precast Tris-Cl 10% SDS-polyacrylamide Ready-gels (Biorad Laboratories, Hercules, CA) were used. To prepare the separating gel, 4.05 ml of H$_2$O, 2.5 ml of 1.5 M Tris-Cl, pH 8.8, 0.1 ml of 10% (w/v) SDS, and 3.3 ml of 30% bis-acrylamide were pipetted into a 100 ml vacuum flask, the solution mixed gently by swirling, degassed for 15 minutes under vacuum and polymerized by addition of 50 $\mu$l of 10% (w/v) ammonium persulfate and 5 $\mu$l of TEMED. The solution was transferred to prepared glass plates with a 10 ml syringe and an 18-gauge needle. The glass plates were filled to 1 cm below the bottom of the comb, and the acrylamide was layered with water to prevent the top of the gel from drying. The gel polymerized for 1 hour.

To pour the stacking gel, 6.1 ml of H$_2$O, 2.5 ml of 0.5 M Tris-Cl, 100 $\mu$l of
10% (w/v) SDS and 1.3 ml of acrylamide were added to a 100 ml vacuum flask, the solution swirled to mix, degassed under vacuum for 15 minutes and the gel polymerized by addition of 50 μl of ammonium persulfate and 10 μl of TEMED. The water was poured off the top of the polymerized separating gel and the excess water removed by capillary action with a Kim-wipe. The stacking gel was swirled gently to mix and slowly transferred to the top of the separating gel with a 10 ml syringe and an 18-gauge needle. The comb was carefully lowered into the stacking gel and left at room temperature for 1 hour to polymerize. A 5X stock of running buffer was prepared which contained 9 g Tris base, 43.2 g glycine, and 3 g SDS in 600 ml of H₂O. For each experiment, 66 ml of the 5X running buffer was diluted with 330 ml of H₂O. The gel was loaded with 15 μg of microsomal protein or 5 to 7.5 μg protein for standards (Gibco-BRL unstained high markers or Biorad Kaleidoscope markers) after addition of an equal volume of sample buffer (4 ml of H₂O, 1 ml of 0.5 M Tris-Cl, pH 6.8, 0.8 ml of glycerol, 1.6 ml of 10% (w/v) SDS, 0.4 ml of β-mercaptoethanol, 0.2 ml of 0.05% (w/v) bromophenol blue). The gel was run at a constant voltage of 200 volts until the dye front was approximately 0.5 cm from the bottom of the gel, typically 45 minutes. The gel was removed from the glass plates and soaked in cold transfer buffer (25 mM Tris-Cl, 192 mM glycine, and 20% (v/v) methanol) for 30 minutes. In a separate container, a piece of Hybond nitrocellulose membrane (Amersham, Arlington Hts., IL) was soaked in transfer buffer for 30 minutes.
The gel was electroblotted using a Biorad Transblot apparatus. The blot was layered in the following manner: sponge pad, fiber pad, gel, membrane, fiber pad, sponge pad, each soaked in transfer buffer prior to use. The fiber pads were cut to the same size as the gel and membrane. The assembled transblot apparatus was placed in the chamber of the Transblot apparatus containing ice and transfer buffer with the membrane toward the anode. The proteins were transferred from the gel to the membrane at 230 mA constant current for 2 hours. After 2 hours the membrane was removed from the gel and stained with Ponceau S to confirm the transfer of the protein. The membrane was dried at room temperature for 30 minutes, soaked in H$_2$O for 5 minutes, soaked in Ponceau S (Sigma, 5 ml concentrate in 45 ml of H$_2$O) for 10 minutes, then rinsed in H$_2$O to remove excess stain. The location of the standards on the membrane was often marked with a pencil.

10. Immunoblotting:

The membrane with the electrotransferred proteins was blocked with phosphate buffered saline (PBS, Gibco-BRL) containing 5% skim milk for 1 hour at room temperature in a rotating cylinder to maintain constant contact with the blocking agent. The membrane was blocked to decrease non-specific binding of the primary antibody. The blocking solution was poured off the membrane, discarded, and replaced with 10 ml of the anti-P450 primary antibody solution. The primary antibody, a goat anti-rat 2C11
polyclonal IgG (Daiichi Chemical Corporation through Gentest, Woburn, MA), was diluted 1:5000 in PBS containing 0.5% skim milk. The membrane was soaked in the antibody solution for 1 hour at room temperature. After 1 hour the primary antibody solution was poured off and the membrane washed for 5 minutes at room temperature in 20 ml of PBS containing 0.1% Tween 20. The wash was repeated three times to remove all of the primary antibody. The secondary antibody, an alkaline phosphatase conjugated anti-goat IgG (Sigma), was diluted 1:30,000 with PBS containing 0.5% skim milk and added to the membrane. The secondary antibody was incubated with the membrane for 1 hour at room temperature, poured off the membrane and discarded, and the membrane washed four times with PBS contained 0.1% Tween 20 as described previously. To prepare the 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution, one 25 mg tablet was dissolved in 0.5 ml of DMSO. To prepare the nitro blue tetrazolium (NBT) stock solution, one 10 mg tablet was dissolved in 1 ml of water. To prepare the substrate solution for the immunoblot, 990 µl of NBT stock solution was combined with 30 ml of substrate buffer (0.1 M Tris, 100 mM sodium chloride, 5 mM MgCl₂, adjusted to pH 9.5 with 10 N NaOH) in a 100 ml glass bottle and mixed by inverting. A 99 µl portion of the BCIP stock solution was added and the bottle inverted to mix. The location of the antibody conjugated proteins was determined by placing the membrane in a small container and soaking in the NBT/BCIP substrate solution at room temperature. When the bluish-purple
bands developed on the membrane, the substrate solution was poured off, discarded, and the membrane washed with water. The membrane was transferred to a piece of filter paper and dried in the dark.

Analytical methods for the separation, identification, and quantification of DPH and its metabolites:

Analysis of DPH metabolism by reverse phase HPLC:
The metabolites of DPH from incubations of DPH with microsomes of yeast expressing cytochrome P450s were resolved by injection of the samples (130-250 μl dissolved in mobile phase) into an HPLC (Hewlett Packard 1090 or 1050) with an Ultramex 3 μm, 4.6 x 250 mm, C18 column (Phenomenex, Torrance, CA). The metabolites were separated by gradient elution with 5 mM phosphate buffer pH 3.0 (A) and ACN (B). The following HPLC gradient conditions were used: An initial gradient from 15% to 23% B in 35 min, then to 29% B at 40 min, a linear increase to 100% B at 45 min, then isocratic at 100% B until 50 min, and a linear gradient to 15% B at 52 min. The column was equilibrated at 15% B until 60 min. The flow rate was 1.2 ml/min. The metabolites were identified by coelution with authentic standards obtained from Sigma (p-HPPH, m-HPPH, catechol) or isolated from rat urine (DHD). The o-HPPH was a gift from Dr. Kenneth Dudley (Dept. Pharmacology, Univ. North Carolina, Chapel Hill, NC). Typical retention times with the Ultramex
column for DHD, \( p \)-HPPH, \( m \)-HPPH, and DPH were 6, 18.5, 22, and 40 min, respectively. The \( ^{14} \)C labelled metabolites were detected with a Packard 525A radioactivity detector (Packard, Meridien, CT) with an 800 \( \mu l \) glass solid scintillant flow cell. An HPLC chromatogram of the standards separated with the Ultramex column is shown in Figure 5.

A Hewlett Packard 1090M HPLC, a 4.5 mm x 25 cm, 5 \( \mu m \), C18 column from III Supplies Co. (Meridien, CT), and a Packard Flow-one \( \beta \) radioactivity detector with a 2 ml liquid flow cell were used for analysis of metabolites from cytochrome P450 enzymes expressed in COS-1 cells and cytochrome P450 enzymes purified from human livers. The metabolites were measured with the Packard Flow-one \( \beta \) radioactivity detector using an HPLC flow rate of 1.2 ml/min and Scintiverse LC (Fisher), as the scintillant, at a flow rate of 5 ml/min.

For purification of the DHD metabolite, a Nucleosil 4.6 mm x 25 cm, 5 \( \mu m \), C18 column (Alltech, Deerfield, IL) was used, but variation between columns in the resolution of metabolites resulted in a change to the 4.5 mm x 25 cm, 5 \( \mu m \) C18 column from III Supplies Co. (Meridien, CT). The HPLC conditions for the III column were as follows. The mobile phase consisted of (A) 5 mM phosphate buffer (pH 3.0) and (B) ACN with a flow rate of 1.2 ml/min. The metabolites were separated by the following gradient: 15% B initially to 23%
Figure 5. HPLC separation of DHD, CAT, p-HPPH, m-HPPH, o-HPPH and DPH:
DHD, CAT and o-HPPH together with one nmole of p-HPPH, m-HPPH, and DPH were injected into a Hewlett Packard 1050 and separated using an Ultramex 3 μm, 25 cm x 4.6 mm, C18 column. Typical retention times for the standards were 6, 12, 18.5, 22, 30 and 40 min for DHD, CAT, p-HPPH, m-HPPH, o-HPPH, and DPH, respectively.
B at 35 minutes, a linear increase to 41% B at 50 minutes, a linear increase to 100% B at 59 min, then isocratic at 100% B until 69 minutes. The solvent was changed with a linear gradient to 15% B at 71 minutes and equilibrated at 15% B until 81 minutes. Typical uv retention times for the column from III Supplies Co. with this gradient were 8, 17, 24.5, 28.5, 32.5, and 45 minutes for DHD, CAT, p-HPPH, m-HPPH, o-HPPH, and DPH, respectively. Retention times of the radioactive peaks with the Flow-one β radioactivity detector were approximately 1 minute later. III Supplies no longer produces HPLC columns, so Ultramex C18 columns are now used.

Purification of $^{14}$C-DPH by solvent extraction:
The [4-$^{14}$C]-5,5-diphenylhydantoin (Lots 2824-087 and 2955-154, specific activities 47.2 and 53.1 μCi/μmole, respectively) from New England Nuclear (Boston, MA) was tested for radiochemical purity by reverse phase HPLC. The DPH typically had greater than 99% radiochemical purity; however, the impurities eluted from the HPLC column at the same retention times as p-, m-, and o-HPPH, as shown in Figure 6. The radiolabelled DPH was purified prior to use in metabolic studies by extraction with phosphate buffer and n-chlorobutane. A 50 μCi portion of [14C]-DPH in 0.5 ml of ethanol was added to 40 ml of n-chlorobutane in a 50 ml conical vial. The tube was capped and shaken for 10 minutes on a horizontal shaker. A 5 μl aliquot was counted to confirm the amount of radioactivity in the tube. Four ml of 0.1 M phosphate
buffer (pH 6.8) was added, the tube shaken for 10 minutes on the horizontal shaker, and centrifuged at 1500 x g for 10 minutes to separate the phases. The 40 ml of n-chlorobutane was transferred to a clean tube and another 4 ml of phosphate buffer added. The extraction of the polar metabolites into the phosphate buffer was repeated twice for a total of three extractions. The 40 ml of n-chlorobutane was transferred to a round bottom flask and evaporated to dryness using a rotary evaporator. The residue in the round bottom flask was redissolved with absolute ethanol and transferred to a glass conical vial using several 1 ml portions. The ethanol was dried with a gentle stream of nitrogen, the purified DPH redissolved in 1 ml of ethanol, and the vial sealed with a Teflon-lined screwcap. The concentration of radioactivity was determined by counting two 2 µl aliquots. The purity was tested by reverse phase HPLC. Recovery of ¹⁴C-DPH after purification was about 85%.

Isolation and identification of the DHD metabolite from the urine of the rat:
The DHD was purified from the urine of a 271.5 g Sprague-Dawley male rat pretreated for 3 days with 75 mg/kg phenobarbital (Sigma) prior to dosing with DPH. The rat was dosed i.p. with 40 mg/kg ¹⁴C-DPH (2.37 µmole/µCi), placed in a metabolism cage, and urine collected on ice for 36 hours. The rat excreted 12.36 µCi in 9.73 ml of urine during the first 24 hours. This 24 hour urine collection was used for isolation of the DHD. To determine the
profile of metabolites in urine, 200 μl portions of the 24-hour urine were incubated at 37°C for 20 hours with 200 μl of 20,000 U/ml β-glucuronidase in 0.2 M sodium acetate buffer (pH 4.9) or with 200 μl of 0.2 M sodium acetate buffer alone. The urine was extracted by addition of 600 μl ACN, vortexed, centrifuged at 1800 x g, and transferred to 1.5 ml eppendorf tubes. The ACN extracts were dried in a centrifugal evaporator and redissolved in 300 μl of 80% H2O/20% ACN. The recovery of radiolabel from these incubations was approximately 87%. To determine the profile of metabolites, shown in Figure 7, 150 μl portions were injected on the HPLC. The DHD was separated by gradient elution from a Nucleosil C18, 5 μm, 4.5 mm x 25 cm HPLC column (Alltech, Deerfield, IL) with a mobile phase of (A) phosphate buffer, pH 3.0 and (B) ACN. The gradient conditions were as follows: The initial mobile phase, 15% B, was changed to 23% B in 35 minutes with a linear gradient, to 41% B at 50 minutes with a linear gradient, increased to 100% B at 59 min with a linear gradient and remained isocratic at 100% B until 69 minutes. The mobile phase returned to 15% B at 71 minutes with a linear gradient and the column equilibrated at 15% B until 81 minutes. With these conditions, the DHD, CAT, p-HPPH, m-HPPH, o-HPPH and DPH eluted at 12, 19, 29, 33, 42, and 48 minutes, respectively. The DHD in each of the samples accounted for approximately 27% of the total radioactivity.
Figure 6. Purification of $^{14}$C-DPH: Prior to purification by solvent extraction, the DPH contained radioactive peaks which coeluted with $p$-, $m$-, and $o$-HPPH as shown in Panel A. The DPH was transferred to n-chlorobutane and the radiochemical impurities removed by extracting 3 times with 0.1 M phosphate buffer (pH 6.8) as shown in Panel B. Approximately 1 $\mu$Ci of DPH was injected into the HPLC and analyzed with a 5 $\mu$m, C18 HPLC column from III Supplies Co.
To isolate the DHD a 1.5 ml portion of the 24-hour urine (1.9 μCi) was added to 1.5 ml of 20,000 U/ml β-glucuronidase in 0.2 M sodium acetate buffer (pH 4.9), heated at 37°C for 20 hours, extracted with 9 ml of ACN, lyophilized, and the residue reconstituted in 4.5 ml of mobile phase. The DHD was collected by twelve 250 μl injections into an HPLC (1.27 μCi total radioactivity, approximately 0.342 μCi (0.811 μmole) DHD) and separated by gradient elution with a Nucleosil column, as described previously. The eluent containing the DHD, monitored at 210 nm, was collected in a flask, lyophilized, and the residue reconstituted in 1 ml of 80% H₂O/20% ACN. The reconstituted sample contained 0.295 μCi or 0.699 μmole of DHD. A 4 μg portion of the reconstituted DHD was injected onto the HPLC column and approximately 50% of the radiolabel was DHD, while p- and m-HPPH accounted for 18 and 20%, respectively; therefore, the 0.295 μCi from the original purification corresponded to 0.148 μCi or 0.351 μmole of DHD. The p- and m-HPPH was probably formed by acid catalyzed loss of water from the DHD in the pH 3.0 mobile phase during collection and lyophilization as described by Chang (43). The DHD was purified by reinjection on the Nucleosil C18 column with a mobile phase of 80% H₂O/20% ACN, collection of the eluent containing the DHD, lyophilization, and reconstitution in 1 ml of ACN. The amount of DHD purified was 0.107 μCi (0.254 μmole, 72.6 μg).
The DHD was identified by direct probe mass spectrometry with positive ion chemical ionization with methane gas. The mass spectrum of the DHD is shown in Figure 8. The base peak was 269 m/z, corresponding to [M - H\_2O] + H with methane adducts (C\_2H\_5 and C\_3H\_5) at m/z 297 and 309. The molecular ion, [M] + H, at m/z 287 was identified, as well as methane adducts of this ion at m/z 315 and 327.

**Metabolism of DPH by yeast microsomes expressing isozymes of cytochrome P450:**

Incubations contained 15 µg dilaurylphosphatidylcholine (DLPC), 500 units of NADPH-oxidoreductase, 1 mg of microsomal protein of yeast expressing the cytochrome P450, 1 mg of microsomal protein of yeast expressing cytochrome b\_6, 25 µM DPH (0.664 µCi) or 50 µM DPH (1.33 µCi), and 50 mM HEPES, 1.5 mM MgCl\_2, 0.1 mM EDTA (pH 7.4) in a final volume of 500 µl.

A 200 µl aliquot of a 5 mg/ml stock solution of DLPC in chloroform was pipetted into a 12 x 75 mm glass tube, dried under a gentle stream of nitrogen and redissolved in 1 ml of water. The redissolved lipid was sonicated on ice with a Tekmar 50 W instrument set at 40 with four 15-second bursts. The final concentration of the stock DLPC was 1 mg/ml.
Figure 7. Profile of the metabolites of DPH from rat urine: Urine from a rat injected ip with 40 mg/kg $^{14}$C-DPH (2.37 μmole/μCi) was incubated with an equal volume of 20,000 U/ml β-glucuronidase in 0.2 M sodium acetate buffer (pH 4.9) or an equal volume of buffer alone for 20 hours at 37°C in a shaking water bath (Panel A and B, respectively). The urine was extracted with ACN, lyophilized, reconstituted and analyzed by HPLC. The DHD eluted at 12 minutes and accounted for 27% of the radioactivity in both incubations.
Figure 7.

(A) Radioactivity (1000 dpm FS) over time (minutes):
- A
- DHD
- p-HPPH
- O-MeCAT
- m-HPPH
- CAT
- DPH

(B) Radioactivity (1000 dpm FS) over time (minutes):
- B
- polar peak
- DHD
- p-HPPH
- O-MeCAT
- m-HPPH
- CAT
- DPH
Figure 8. Mass spectrum of DHD purified from rat urine: The DHD, purified from the urine of a rat injected ip with 40 mg/kg $^{14}$C-DPH (2.37 µmole/µCi) by HPLC, was identified by direct probe mass spectrometry with positive ion chemical ionization with methane gas. The base peak of m/z 269 corresponds to [M-H$_2$O] + H, with methane adducts (C$_2$H$_5$ and C$_3$H$_6$) at m/z 297 and 309. The m/z 287 corresponds to the molecular ion [M] + H, with methane adducts at m/z 315 and 327. The fragments m/z 175 and 253 correspond to [M-2OH] + H and M-C$_6$H$_7$O$_2$, respectively.
The reactions were as follows: A 15 µl aliquot of DLPC was added to prechilled, glass, 12 x 75 mm test tubes. Microsomes of lymphoblastoid cells representing 500 units of NADPH-oxidoreductase (Gentest, Woburn, MA) were added and the tubes vortexed. Microsomes of yeast expressing cytochrome P450 representing 1 mg of microsomal protein were added and the tubes vortexed. Microsomes of yeast expressing cytochrome b₅ were added and the tubes vortexed. The tubes were incubated at 37°C for 5 minutes in a water bath and placed on ice while the buffer and substrate were added. DPH was added to the incubations as a 25 µl aliquot of 0.5 or 1 mM stock solutions for incubations with 25 µM (0.664 µCi) and 50 µM (1.33 µCi) final concentrations, respectively. Buffer, 50 mM HEPES, 1.5 mM MgCl₂, 0.1 mM EDTA (pH 7.4), was added to the tubes to adjust the total volume of the incubations to 500 µl. The tubes were vortexed after addition of the DPH and buffer. The tubes were incubated for 2 minutes at 37°C, and the reactions started by adding 50 µl of 10 mM NADPH. In control incubations, 50 µl of buffer was added. The reactions were stopped after 2 hours by adding 1.5 ml of ACN, vortexing, and centrifuging at 1800 x g to pellet the proteins. The entire supernatants, which contained DPH and metabolites, were transferred to clean glass tubes and evaporated to dryness under a gentle stream of nitrogen. The protein pellets were resuspended in 500 µl of ACN, vortexed to extract residual radioactivity, and centrifuged to pellet the protein. The ACN was transferred to the tube containing the original supernatant and
evaporated to dryness with a gentle stream of nitrogen. The washes were repeated until the extractable radioactivity was less than twice background, or approximately 90 dpm, typically 3 washes. The residues from the evaporated supernatants were often yellowish and gummy, a result of the glycerol in the buffer used to resuspend the microsomes of yeast expressing cytochrome P450 and cytochrome b5 which is extracted from the incubation with the ACN. The residues were reconstituted by adding 60 μl of ACN, 60 μl of water, then 180 μl of water and vortexing for 2 minutes between each addition. The final volume was often slightly more than 300 μl due to the presence of the glycerol carried by the ACN and water into the residues. A 250 μl portion of the redissolved sample was injected for analysis by HPLC with detection of the radioactive metabolites with a flow-through radioactivity detector. The recovery of radioactivity for these incubations was 93.8 ± 7.5% (n = 14). In initial experiments, the supernatants were dried in a centrifugal evaporator, the residues reconstituted in 150 μl mobile phase and 130 μl analyzed by HPLC. Recovery of radiolabel was variable and poor with these conditions. The recoveries ranged from 45 to 96% (74 ± 16%, n = 10). Radiolabel was found in the evaporator after drying these samples. Therefore, the method was changed to drying the supernatants with nitrogen and reconstituting the residues in 300 μl of mobile phase.
Metabolism of DPH by microsomes of yeast expressing isozymes of cytochrome P450 with added microsomal epoxide hydrase:

For incubations of DPH with cytochrome P450 enzymes expressed in yeast and microsomal epoxide hydrase, the reaction conditions described above were used with the following modification. Incubations of DPH with yeast expressing cytochrome P450 2C9 were conducted with 0.2 mg/ml of microsomal protein from lymphoblastoid cells expressing microsomal epoxide hydrase (Gentest, Woburn, MA), added to the tubes following the addition of cytochrome b$_5$. All other conditions were as described.

Metabolism of DPH by cytochrome P450 2C8 and cytochrome P450 2C9 purified from human liver:

Cytochrome P450 enzymes 2C8 and 2C9 were obtained from Drs. J. Raucy (Toxicology Program, University of New Mexico, Albuquerque, NM) and J. Lasker (Department of Biochemistry, Mt. Sinai School of Medicine, NY, NY) to study the metabolism of DPH. The methods used to purify these two enzymes from human liver have been described (134). Cytochrome b$_5$ and NADPH-oxidoreductase were also provided by Drs. Raucy and Lasker. The concentrations of the stock solutions of cytochromes 2C8, 2C9, and b$_5$, and NADPH-oxidoreductase were 3.25 nmol/ml, 8.45 nmol/ml, 15.4 nmol/ml and 10,700 units/ml, respectively. The incubations with DPH contained 50 pmole of purified P450, 500 units of NADPH-oxidoreductase, 30 µg/ml of DLPC and
200 pmoles cytochrome b$_5$ in a volume of 121 $\mu$l.

In order to prepare the lipid, a 200 $\mu$l aliquot of a 5 mg/ml solution of DLPC in chloroform was pipeted into a 12 x 75 mm glass tube, dried under a gentle stream of nitrogen, redissolved in 1 ml of H$_2$O, and sonicated for 15 seconds with a Tekmar 50W sonicator set at 40. The sonication was repeated three times at 1-minute intervals. During sonication, the lipid solution became clear.

To reduce variability in the replicate incubations, the lipid and proteins for 4 incubations (2C8 and 2C9 incubations) or 1 incubation (no P450) were combined in prechilled 12 x 75 mm glass tubes, vortexing between each addition as follows: 1. The tube with lipid and protein for the incubation without P450 contained 3.6 $\mu$l of DLPC, 47 $\mu$l of reductase, 0 $\mu$l of P450, and 13 $\mu$l of cytochrome b$_5$; 2. The tube with lipid and protein for 4 incubations with P450 2C8 contained 14.4 $\mu$l of DLPC, 187 $\mu$l of reductase, 61.5 $\mu$l of P450 2C8, and 52 $\mu$l of cytochrome b$_5$; 3. The tube with lipid and proteins for 4 incubations with P450 2C9 contained 14.4 $\mu$l of DLPC, 187 $\mu$l of reductase, 24 $\mu$l of P450 2C9, and 52 $\mu$l of cytochrome b$_5$.

For each incubation, 63.6, 79 $\mu$l or 69 $\mu$l of the combined lipid and proteins were pipetted into prechilled, glass 12 x 75 mm tubes for incubations of DPH
with no P450, P450 2C8, and P450 2C9, respectively. The tubes were incubated at 37 °C for 5 minutes, then placed on ice until the buffer and the substrate were added. Thirty μl of DPH as a 1 mM dose solution (248 μM, 1.47 μCi final concentration) and 0.1 M phosphate buffer (15.4, 0 and 10 μl to the no P450, 2C8, and 2C9 tubes, respectively) were added to each tube. The total incubation volume was 121 μl. The incubations were preincubated for 2 minutes at 37 °C, and the reactions started by addition of 12 μl of 10 mM NADPH. The reactions were stopped after 2 hours by addition of 300 μl of ACN, vortexed for 5 minutes, and centrifuged at 14,000 x g for 10 minutes. The supernatants, which contained the metabolites and DPH, were transferred to clean eppendorf tubes and dried in a centrifugal evaporator. The metabolites were measured by HPLC as described with a III C18 HPLC column.

Metabolism of DPH by human liver microsomes:
Microsomes from human livers were purchased from Keystone Skin Bank (Exton, PA). The livers for preparation of these microsomes are typically surgical waste or donor organs which did not match a recipient. For these experiments the microsomes were from livers of 55- and 62-year-old females. For experiments in the metabolism of DPH, these were assumed to be representative of the general population. Neither donor had significant medical problems, and neither had a history of drug or alcohol abuse.
Incubations contained 2 mg/ml of microsomal protein, 125 \( \mu \text{M} \) (0.664 \( \mu \text{Ci} \)) or 250 \( \mu \text{M} \) DPH (1.33 \( \mu \text{Ci} \)) and 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.4) in a final volume of 100 \( \mu \text{l} \). The reactants were preincubated for 2 minutes at 37°C, and the reactions started by addition of cofactors (0.5 mM NADP, 10 mM glucose-6-phosphate, 1.25 units glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride, final concentrations). The incubations were stopped after 2 hours by addition of 300 \( \mu \text{l} \) of ACN, vortexed, and centrifuged at 14,000 x g for 5 minutes to precipitate the proteins. The supernatants, which contained DPH and the metabolites, were transferred to a clean eppendorf tube and evaporated completely under a gentle stream of nitrogen. The protein pellets were washed by addition of 0.5 ml of ACN, vortexed, centrifuged at 14,000 x g for 5 minutes, and the ACN wash combined with the original supernatant and evaporated with a stream of nitrogen. The protein was washed with ACN until the extractable radioactivity was less than twice the background of the scintillation counter, typically less than 90 dpm. The residue from the supernatant and washes was reconstituted by adding 60 \( \mu \text{l} \) of ACN, 60 \( \mu \text{l} \) of H\(_2\)O, then 180 \( \mu \text{l} \) of H\(_2\)O, vortexing for 2 minutes after each addition. The reconstituted samples were analyzed by HPLC with an Ultramex C18 column.

Inhibition by sulfaphenazole of DPH metabolism in human liver microsomes:
Sulfaphenazole was added to incubations with human liver microsomes to
determine the effect on the metabolism of DPH. Incubations contained 2 mg/ml of microsomal protein, 125 μM DPH (0.664 μCi), 0, 1, 10, or 50 μM sulfaphenazole, and 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.4) in a final volume of 100 μl. The tubes were preincubated at 37°C for 2 minutes and the reactions started with addition of cofactors (0.5 mM NADP, 10 mM glucose-6-phosphate, 1.25 units glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride, final concentration). Reactions were stopped after 2 hours by adding 300 μl of ACN and the metabolites analyzed as described previously. Recovery of label in experiments with human liver microsomes was 100.7 ± 6.4% (n = 14).

Metabolism of styrene oxide by microsomal epoxide hydrase:
To measure the activity of microsomal epoxide hydrase expressed in lymphoblastoid cells (Gentest, Woburn, MA), the conversion of styrene oxide to styrene glycol was measured. Styrene oxide was added as 50 μl of a 1 mM stock solution, prepared by adding 1.14 μl styrene oxide to 10 ml of 50/50 ACN/H2O. Incubations contained 0.2 mg/ml of microsomal epoxide hydrase, 100 μM styrene oxide and 50 mM HEPES, 1.5 mM MgCl2, 0.1 mM EDTA buffer in a final volume of 500 μl. Reactions were incubated at 37°C for 10, 20, or 30 minutes and stopped by placing the tubes on ice. Control incubations did not contain microsomal epoxide hydrase and were incubated at 37°C for 30 minutes. Styrene oxide was extracted from the incubations
by adding 1 ml of hexane to the reactions, vortexing, and centrifuging at 14,000 x g for 5 minutes. The hexane was discarded and the aqueous phase extracted with two more 1 ml portions. The styrene glycol was extracted by addition of 1 ml of ethyl acetate to the aqueous phase, vortexing, centrifuging at 14,000 x g for 5 minutes, and transferring the ethyl acetate to a clean tube. The extraction was repeated twice and the ethyl acetate extracts combined and dried under a gentle stream of nitrogen. The residue was dissolved in 500 µl of mobile phase and vortexed for 30 minutes. A 150 µl portion of the sample was injected into the HPLC, and the metabolites separated with an Ultramex, C18, 3 µm, 4.6 mm x 25 cm column with a Hewlett Packard 1050 HPLC. The mobile phase was 95% phosphoric acid/5% ACN, the flow rate 1.2 ml/min and the peaks detected at 210 nm. The retention time of styrene glycol was 17.6 min.
RESULTS

Incubation of DPH with human liver microsomes:
The profile of metabolites formed by incubation of DPH with microsomes from human liver is shown in Figure 9. The microsomes produced p-, m. and o-HPPH as well as a polar peak with a retention time corresponding to the DHD. The m- and o-HPPH and more polar products and a portion of the p-HPPH may be formed by autooxidation of the iron-oxygen complex of the cytochrome P450 enzymes, which may produce superoxides, hydrogen peroxide, hypochlorous acid, and hydroxyl radical. No metabolism of DPH occurred without cofactors.

Incubation of DPH with cytochrome P450s 2C8 and 2C9 purified from human liver:
Cytochrome P450 2C9 metabolized DPH to 123 ± 20 pmole p-HPPH/50 nmole cytochrome P450 in 2 hours (n=3) as shown in Figure 10. Cytochrome P450 2C8 purified from human liver metabolized DPH to 19 ± 0 p-HPPH/50 nmole cytochrome P450 in 2 hours (n=3) as depicted in Figure 11. The recovery of radiolabel from these incubations was 98.7 ± 6.8% (n = 6). The kinetics of the hydroxylation reactions were not measured owing
Figure 9. Metabolism of DPH by human liver microsomes: Human liver microsomes, 2 mg/ml, were incubated with 250 μM DPH (1.33 μCi) and cofactors for 2 hours at 37°C. The metabolites were separated with a HP1050 HPLC using an Ultramex 3 μm, C18 column. The radiolabelled metabolites were detected with a Packard 525A flow-through radioactivity detector.
Figure 10. Metabolism of DPH by cytochrome P450 2C9 purified from human liver: DPH (248 μM, 1.47 μCi) was incubated with 50 pmole of cytochrome P450 2C9, 500 units of reductase, 200 pmole of cytochrome b₅, and 30 μg/ml DLPC in a total volume of 121 μl. The reactions were started with 1 mM NADPH and incubated for 2 hours at 37°C. The metabolites were measured by HPLC with a III, 5 μm, C18 column and a flow-through radioactivity detector.
Figure 11. Metabolism of DPH by cytochrome P450 2C8 purified from human liver: DPH (248 μM, 1.47 μCi) was incubated with 50 pmole of cytochrome P450 2C8, 500 units of reductase 200 pmole of cytochrome b₅, and 30 μg/ml DLPC in a total volume of 121 μl. The reactions were started with 1 mM NADPH and incubated for 2 hours at 37 °C. The metabolites were measured by HPLC with a III, 5 μm, C18 column and flow-through radioactivity detector.
to the small quantities of available protein.

**Metabolism of DPH by isozymes of cytochrome P450 expressed in COS-1 cells:**

In order to more specifically characterize the metabolism of DPH by cytochrome P450s 2C8 and 2C9, COS-1 cells were transiently transfected with the cDNA encoding these enzymes. The metabolism of DPH by COS-1 cells transfected with the expression plasmid 2C9-pcDNA/AMP is shown in Figure 12. In a representative experiment, the cytochrome P450 enzyme formed 49 pmoles p-HPPH (mean of 2 determinations). However, no additional metabolites were detected, unlike human liver microsomes which also produced polar peaks coeluting with DHD, m-, and o-HPPH. The limit of sensitivity for the DHD was approximately 4 pmoles. In contrast, COS-1 cells transfected with the expression plasmid 2C8-pcDNA/AMP failed to metabolize DPH added to the media, as shown in Figure 12. There was no evidence of metabolism of DPH by untransfected COS-1 cells. The recovery of radiolabel in this experiment was approximately 84 ± 8% (n=6).

**Incubation of DPH with microsomes of S. cerevisiae expressing cytochrome P450 2C9, cytochrome P450 2C18, and cytochrome P450 2C8:**

To achieve stable expression of greater quantities of protein than in COS-1 cells, the 2C isozymes were expressed in *S. cerevisiae* and the metabolism
of DPH measured in microsomes from these cells. The profile of metabolites following incubation of DPH with microsomes of *S. cerevisiae* transformed with the expression plasmid 2C9-pRS426-pG1 is shown in Figure 13. Microsomes of yeast expressing cytochrome P450 2C9 formed p-HPPH, but did not form DHD, m-HPPH or o-HPPH. This matched the profile of metabolites with DPH and cytochrome P450 2C9 expressed in COS-1 cells. The sensitivity of the radioactivity detector for DHD was approximately 4 pmole. The activities of cytochrome P450 2C9 for the metabolism of DPH are summarized in Table 1.

The cDNA which encodes cytochrome P450 2C18 was subcloned into the yeast expression vector pRS426-pG1 to study the metabolism of DPH. The profile of metabolites following incubation of DPH with microsomes of yeast expressing cytochrome P450 2C18 is shown in Figure 14. Like cytochrome P450 2C9, this isozyme produced p-HPPH but no additional metabolites. The activities of cytochrome P450 2C18 for the metabolism of DPH are summarized in Table 1. A representative chromatogram of microsomes of yeast expressing cytochrome P450 2C18 and DPH incubated at 37°C for 2 hours in the absence of cofactors is depicted in Figure 15.

As shown in Figure 16, microsomes of yeast transformed with the expression plasmid CYP2C8-pRS426-pG1 did not metabolize DPH. The expression of
Figure 12. Metabolism of DPH by CYP2C9 and CYP2C8 expressed in COS-1 cells: COS-1 cells in a 60 mm² plate were transfected with a solution containing 5 µg of plasmid DNA and 30 µl of Lipofectin for 16 hours. DPH (1.5 µCi) was added to the cell media and incubated for 30 hours. The metabolites of DPH in the cell media were measured by HPLC using a flow-through radioactivity detector. Panels A and B represent COS-1 cells expressing 2C9 and 2C8, respectively.
Figure 12. Radioactivity (1000 dpm FS) vs. Time (minutes)

Panel A: 
- Radioactivity peak at 20 minutes
- Phenobarbital

Panel B: 
- Radioactivity peak at 30 minutes
- Phenobarbital

PM-HPPH peak at 40 minutes
cytochrome P450 2C8, 2C9, and 2C18 in microsomes of yeast was examined by Western blotting following SDS-PAGE as shown in Figure 17. Both cytochrome P450 2C8 and 2C9 were detected by the anti-2C11 polyclonal antibody, indicating that these proteins were expressed; however, cytochrome P450 2C18 did not cross-react.

The metabolism of [14C]-warfarin by microsomes expressing cytochrome P450 2C8 is currently being studied to ensure that the protein detected by immunoblotting is catalytically active.

**Incubation of DPH with expressed cytochrome P450 2C9 and microsomal epoxide hydrase:**

Human microsomal epoxide hydrase was added to incubations with DPH and yeast microsomes expressing cytochrome P450 2C9 to determine the effect on the profile of metabolites. The activity of the microsomal epoxide hydrase was confirmed by measuring the conversion of styrene oxide to styrene glycol as illustrated in Figure 18. The combination of cytochrome P450 2C9 and microsomal epoxide hydrase formed p-HPPH but not DHD, as shown in Figure 19.
Figure 13. Metabolism of DPH by yeast expressing cytochrome P450 2C9: A representative chromatogram of DPH metabolism by yeast expressing cytochrome P450 2C9. Incubations contained 2 mg/ml microsomal protein of yeast expressing cytochrome P450 and cytochrome b₅, 500 units of NADPH-oxidoreductase, and 30 μg/ml dilaurylphosphatidylcholine, and 12.5 μM DPH (0.664 μCi) in 50 mM HEPES buffer containing 1.5 mM MgCl₂ and 0.1 mM EDTA (pH 7.4) in a total volume of 0.5 ml. Reactions, started with 1 mM NADPH, were incubated at 37°C for 2 hours.
Metabolism of DPH by Cytochrome P450 Enzymes Expressed in *S. cerevisiae*

<table>
<thead>
<tr>
<th>Cytochrome P450</th>
<th>( p\text{-HPPH (pmole/mg protein/2 hr)} )</th>
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</thead>
<tbody>
<tr>
<td>2C8</td>
<td>Not Detected</td>
</tr>
<tr>
<td>2C9(^a)</td>
<td>89 ± 8 ((n = 2))</td>
</tr>
<tr>
<td>2C9(^b)</td>
<td>39 ((n = 1))</td>
</tr>
<tr>
<td></td>
<td>48 ± 6 ((n = 4))</td>
</tr>
<tr>
<td>2C18(^b)</td>
<td>48 ± 5 ((n = 4))</td>
</tr>
<tr>
<td></td>
<td>28 ± 2 ((n = 4))</td>
</tr>
</tbody>
</table>

Microsomes of yeast expressing cytochrome P450 enzymes were incubated with DPH for 2 hours at 37 °C. Metabolites of DPH were measured by HPLC as described in Methods. No metabolites were detected in incubations without added cofactors. \(^a\) The concentration of DPH was 50 \(\mu M\). \(^b\) The concentration of DPH was 25 \(\mu M\).

Table 1
Figure 14. Metabolism of DPH by yeast expressing cytochrome P450 2C18: Representative chromatogram of DPH metabolism by yeast expressing cytochrome P450 2C18. Reaction conditions were as described in Figure 13.
Figure 15. Metabolism of DPH by yeast expressing cytochrome P450 2C18 without cofactor: Representative chromatogram of the incubation of microsomes of yeast expressing cytochrome P450 2C18 with the reaction conditions described in Figure 13 without 1 mM NADPH.
Figure 16. Metabolism of DPH by yeast expressing cytochrome P450 2C8: Representative chromatogram of DPH metabolism by yeast expressing cytochrome P450 2C8. Reaction conditions were as described in Figure 13.
Figure 17. Western blot of cytochrome P450 enzymes expressed in yeast: Microsomal proteins of yeast expressing cytochrome P450s 2C8, 2C9 or 2C18 were separated by SDS-polyacrylamide electrophoresis and were blotted with an antibody against rat cytochrome P450s 2C6 and 2C11. Each lane contained 15 µg of protein. The molecular weight of cytochrome P450s 2C8, 2C9, and 2C18 are 50 kD, 55 kD, and 55 kD, respectively. The arrow indicates a band of cross-reactivity toward proteins in the yeast.
Figure 18. Metabolism of styrene oxide by microsomal epoxide hydrase: Microsomes of lymphoblastoid cells expressing microsomal epoxide hydrase, obtained from Gentest (Woburn, MA), were incubated with 100 μM styrene oxide at 37 °C. Incubations were stopped at 10, 20 and 30 minutes and the styrene glycol was extracted with ethyl acetate and measured by HPLC.
Figure 19. Metabolism of DPH by cytochrome P450 2C9 expressed in yeast and microsomal epoxide hydrase: The metabolism of DPH by cytochrome P450 2C9 expressed in yeast was measured in the presence of 0.2 mg/ml of microsomes expressing microsomal epoxide hydrase. The conditions for the reactions were as described in Figure 13. The metabolites were separated by HPLC and measured with a flow-through radioactivity detector.
Incubation of DPH with human liver microsomes and sulfaphenazole, an inhibitor of the cytochrome P450 2C subfamily:

Although other cytochrome P450s may metabolize DPH to \( p \)-HPPH and DHD through an epoxide, the formation of \( p \)-HPPH but not DHD by cytochrome P450 enzymes from the 2C subfamily lead to the hypothesis that these metabolites were formed independently. The profile of metabolites following incubation of DPH with human liver microsomes and cofactors is shown in Figure 20. The microsomes formed \( 99 \pm 10 \) pmole \( p \)-HPPH and \( 31 \pm 6 \) pmole of polar peaks which coeluted with DHD. The profile of metabolites in the presence of \( 10 \) \( \mu \)M sulfaphenazole is also shown in Figure 20. In the presence of the inhibitor, the microsomes formed \( 37 \pm 1 \) pmole \( p \)-HPPH and \( 34 \pm 7 \) pmole of the polar peaks. Values are the mean and standard deviation of duplicate values. The formation of \( p \)-HPPH and the polar peaks in incubations of DPH with microsomes of human liver with concentrations of sulfaphenazole up to \( 50 \) \( \mu \)M is shown in Figure 21. The formation of \( p \)-HPPH was significantly inhibited (ANOVA, \( p = 0.0046 \)), but formation of the polar peaks was unchanged, suggesting that these metabolites were formed independently; otherwise the decreased formation of \( p \)-HPPH should correlate with a decrease in the polar peak(s).
Figure 20. Inhibition by sulfaphenazole of the metabolism of DPH by human liver microsomes: Incubations contained 2 mg/ml microsomal protein, 125 μM DPH (0.664 μCi), and 0 or 10 μM sulfaphenazole (Panel A and B, respectively) in a final volume of 0.1 ml of 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.4). Reactions were started by addition of cofactors and incubated for 2 hours at 37°C. The metabolites were measured by HPLC with a Ultramex 3 μm, C18 column and a flow-through radioactivity detector.
Radioactivity (1000 dpm FS)

Time (minutes)

0 10 20 30 40 50

polar peak

p-HPPH

m-HPPH

o-HPPH

DPH

Figure 20.
Figure 21. Inhibition by sulfaphenazole of the metabolism of DPH by human liver microsomes: Human liver microsomes were incubated with DPH, cofactors, and 0 to 50 μM sulfaphenazole, an inhibitor of cytochrome P450 isozymes in the 2C subfamily. Sulfaphenazole significantly inhibited the formation of p-HPPH (ANOVA, \( p = 0.0046 \)) but did not affect the formation of the polar peak(s).
DISCUSSION

Previous studies, reported after our investigation started, have shown that human cytochrome P450 2C9, expressed in COS-1 cells, metabolized DPH to $p$-HPPH, but these studies failed to separate the metabolites of DPH (129-30).

In our studies, two expression systems were used: COS-1 cells and *S. cerevisiae*. COS-1 cells, which constitutively express NADPH-oxidoreductase and cytochrome $b_5$, seem ideal for screening the metabolism of substrates by individual cytochrome P450 isozymes and were commonly used for expression studies by other investigators when our studies were initiated. However, technical difficulties were associated with expression of cytochrome P450s in COS-1 cells; incubations extended over long periods (up to 72 hours), expression of isozymes of cytochrome P450 was transient, substrates required to confirm the activity of expressed cytochrome P450 2C8 (i.e. warfarin and tolbutamide) decompose spontaneously during the long incubation period to products which interfere with the measurement of hydroxylated metabolites. Moreover, styrene oxide, a substrate used to confirm the activity of cotransfected microsomal epoxide hydrase,
spontaneously hydrates to styrene glycol during long incubations in the plates. The cytochrome P450 concentration in the COS cells on the plates is difficult to determine. DPH binds to proteins in the media and cells, thereby complicating kinetic measurements of individual pathways of metabolism. Consequently, we decided to undertake additional experiments with enzymes expressed in *S. cerevisiae*, which can be transformed stably.

Although Pompon *et al.* showed that diol metabolites were formed when benzo[a]pyrene was incubated with yeast cells coexpressing cytochrome P450 1A1 and epoxide hydrase in the same cell or when benzo[a]pyrene was incubated with microsomes of yeast expressing cytochrome P450 1A1 and microsomes of yeast expressing microsomal epoxide hydrase (118), the DHD metabolite of DPH was not formed by addition of epoxide hydrase to microsomes of yeast expressing 2C9.

Cytochrome P450 2C18, not well studied to date, has now been shown by us to metabolize DPH with the same profile of metabolites as cytochrome P450 2C9. It seemed reasonable to postulate that 2C18 would metabolize DPH considering it metabolizes S-mephenytoin and tolbutamide, substrates which are metabolized by isozymes of the homologous 2C subfamily of cytochrome P450 (90, 93, 94). Our experiments show that cytochrome P450 2C18 may also metabolize DPH in microsomes from human liver,
although the importance of this enzyme in the *in vivo* metabolism of DPH has yet to be determined.

Although in our studies cytochrome P450 2C8 purified from human liver metabolized DPH to *p*-HPPH, COS cells and yeast transfected with cytochrome P450 2C8 failed to do so. This may be attributed to the low amount of enzyme produced by COS and yeast cells. Alternatively, the fraction containing purified cytochrome P450 2C8 isolated from human liver may have been contaminated with cytochrome P450 2C9 or a similar cytochrome P450. Contamination of the purified P450 fractions with cytochrome P450 2C18 or 2C19 is a possibility. These two enzymes had not yet been identified when the cytochrome P450 2C8 and 2C9 were purified, so the purified fractions were not tested for their presence. Furthermore, antibodies used to test the purity of the protein fractions may not crossreact with the other isozymes in the homologous cytochrome P450 2C subfamily. The specific content of 2C8 and 2C9 purified from human liver was approximately 7 and 15 nmol P450/mg protein, respectively (134), which indicated that the 2C8 was less pure than the 2C9. The theoretical specific content is 20 and 18 nmol P450/mg protein for the 2C8 and 2C9, respectively. Data published while these experiments were in progress demonstrated that homogenates of COS-1 cells expressing 2C8 metabolized DPH at a rate which was 3% of the rate of metabolism by COS-1 cells
expressing cytochrome P450 2C9 (92). The experiments described here may not have detected this metabolism because of the small amount of cytochrome P450 2C8 expressed in the COS-1 cells. Conclusions about the metabolism of DPH by cytochrome P450 2C8 await confirmation of the cytochrome P450 2C8 activity with other substrates.

In previous studies, the formation of \( \rho \)-HPPH was attenuated by sulfaphenazole, an inhibitor of 2C enzymes; however, the effect on other metabolites was not measured (92, 130, 135). Addition of 50 \( \mu \)M sulfaphenazole to incubations of DPH and human liver microsomes decreased the formation of \( \rho \)-HPPH by 70% but failed to affect the formation of the polar peak which coeluted with DHD. Moreover, sulfaphenazole did not inhibit the formation of \( m \)- and \( o \)-HPPH. Our data provides evidence that both cytochrome P450 2C9 and 2C18 metabolize DPH to one metabolite, \( \rho \)-HPPH, probably by direct insertion of oxygen and not necessarily by the rearrangement of an epoxide intermediate. The formation of \( \rho \)-HPPH in the absence of any DHD by cytochrome P450 2C9 and 2C18 expressed in \( S. cerevisiae \), further supports this concept. Moreover the obvious dissociation between the inhibition by sulfaphenazole on the production of \( \rho \)-HPPH and DHD is additional evidence that these metabolites are formed independently by different isozymes of cytochrome P450 or other catalytic mechanisms. In the absence of the total recovery of radiolabel in the COS-1 cell experiments
and initial experiments with cytochrome P450s expressed in yeast, the
selective loss of the DHD in the reconstituted samples could not be ruled out.
In later further experiments where recovery is complete, the presence of the
DHD has been ruled out. The ratio of DHD to \( \rho \)-HPH\( \) in incubations of DPH
with microsomes from human liver was approximately 1/3. With this ratio,
together with a sensitivity of 4 pmoles for DHD, the formation of DHD by the
cytochrome P450s expressed in yeast was well within the sensitivity of the
assay.

In spite of our results, it is still possible that other yet unidentified cytochrome
P450 enzymes in the liver may metabolize DPH to both \( \rho \)-HPH and DHD,
perhaps via an epoxide intermediate. Previous studies have shown that the
rates of metabolism of DPH and tolbutamide in microsomes from human liver
correlate closely. Tolbutamide is metabolized by 2C isozymes of cytochrome
P450 (130, 135, 136). Hall et. al., studied the metabolism of DPH,
tolbutamide, and warfarin in microsomes from 14 human livers and found
close correlations between the hydroxylation of tolbutamide, DPH, and
warfarin. However, correlation of these activities to the immunoquantified
level of cytochrome P450 2C9 in these microsomes was poor (137). These
authors attributed this poor correlation to known differences in catalytic
activity of allelic variants of cytochrome P450 2C9 (90, 92, 94). Additionally, these substrates may be metabolized by yet unidentified
cytochrome P450s.

DPH may be metabolized by cytochrome P450 2C19, an isozyme which metabolizes tolbutamide and (S)-mephenytoin. However, when a mutation occurs in the CYP2C19 gene leading to the formation of a truncated enzyme, only (S)-mephenytoin metabolism is abolished. The metabolism of DPH continues to be normal. Therefore, it can be concluded that the metabolism of DPH does not correlate with the S-mephenytoin polymorphism (48) and probably cytochrome P450 2C19 is not a significant isozyme for the metabolism of DPH.

The data shown here using expressed cytochrome P450s 2C9 and 2C18 do not support the hypothesis that an epoxide is an obligate intermediate in the metabolism of DPH to p-HPPH. Our experiments do not rule out the possibility that an epoxide is present in the active site of the enzyme and rearranges to p-HPPH. This epoxide would not, however, be important in forming toxic side-effects. Further work may lead to the characterization of additional cytochrome P450 isozymes in microsomes of human liver which metabolize DPH to p-HPPH and/or DHD.

Our studies, in which p-HPPH is formed directly from DPH from cytochrome P450 2C9 and 2C18, indicate that at least these isozymes are not responsible
for the production of highly reactive and/or toxic products, believed to be responsible for adverse effects of the drug, especially mutagenic, teratogenic, and hypersensitivity effects.

In contrast to heterologous expression systems which expressed individual 2C isozymes, incubations of DPH with microsomes of liver from rats and mice (data not shown) and humans produce polar metabolites, DHD, \( p \)-, \( m \)-, and \( o \)-HPPH. Work with sulfaphenazole, which selectively inhibits isozymes in the 2C subfamily, suggests that some of the metabolites, other than \( p \)-HPPH, are formed by alternate mechanisms such as autooxidation of the cytochrome P450 iron-oxygen complex. The latter process may include the production of superoxide anion, hydrogen peroxide, and hydroxyl radical.

Work in progress is focusing on other enzymes and catalytic systems which may form DHD; it is our intention to study the kinetic properties of the isozymes which metabolize DPH, including cytochrome P450 2C9 and 2C18 with particular reference to the stereoselectivity of these reactions and their activity toward other substrates.


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